RENAL CLEARANCE OF DEXTRAN AS A MEASURE OF GLOMERULAR PERMEABILITY

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Introduction

The mechanisms by which water and solute matter are transported between the different fluid compartments of the body are of the greatest importance for the normal function of the body cells as well as the organ systems. The nutrition of all parts of the organism and the removal of waste products is a matter of transport, and even a slight alteration in these mechanisms may effect serious disturbances in the function of highly specialized cells and tissues. The harmonious combination of these mechanisms is a condition necessary for normal life.

In these transport processes the interfacing membranes or structures may participate actively or passively. An example of the active transport processes is the tubular reabsorption of sugar in the kidney, where the transport is performed by the active, energy-consuming work of the tubular cells. Among the passive transport processes are the interchange of fluid through the capillary walls and the formation of the glomerular fluid in the kidney. These processes require no work from the part of the membrane which here acts purely as a kind of physical filter. In some other cases the transport of a substance through a membrane is a matter of solubility of the substance in the membrane. The transportation of lipid-soluble molecules is an example of this kind of penetration.

Considering the more simple physical filtration or diffusion, it is found that the driving forces here are potential gradients of different character: chemical, electrical, hydrostatic, or surface forces. In order to understand the nature of these transport phenomena it is necessary to know the nature and magnitude of the driving forces, but it is also essential to know the detailed structure and character of the limiting membranes.

The aim of this investigation is to find a way to characterize some of the qualities of an animal membrane, specifically that of the glomerular capillaries of the kidney. The study has been limited to this particular filtration process because the physiology of this organ, due to a large number of investigations, is relatively well known and also because the filtrate elaborated by the kidney is relatively easy to collect and analyze. Furthermore, the mechanism of proteinuria of which only sparse knowledge has been accumulated may be elucidated by a study such as this.

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CHAPTER I

Survey of the Literature

The view generally accepted today of the formation of glomerular fluid is that of Ludwig (1844) and Cushny (1917). They considered the process to be an ultrafiltration of blood plasma through the capillary walls of the glomerular tuft which they believed acted as a semipermeable membrane grossly impervious to plasma proteins. Now a substantial body of evidence has been collected which clearly demonstrates the physical nature of this process. There is no evidence at all to indicate that the glomerulus is capable of carrying out active secretion.

The glomerular fluid so formed passes down through the different segments of the tubuli whose function is to reabsorb most of the water and salts, glucose, amino acids etc. and return them to the blood, avoiding in this manner a depletion of the necessary blood constituents. However, the waste products such as urea etc. are mostly rejected by this reabsorbing mechanism. In addition there also exists an active secretory function of the tubular cells. Concerning these questions of the general physiology of the kidney reference is made to Smith (1951).

As this discussion is limited to the process of glomerular fluid formation, references will here be confined mainly to those authors whose investigations have contributed to the knowledge of this mechanism.

Three ways by which this process may be elucidated can be distinguished. The first is to study the transport through the membrane of low-molecular constituents such as water, electrolytes, sugar, inulin, etc., and to investigate the influence of different factors on this transportation process. The second way to study the filter characteristics is to investigate the passage of macromolecular substances, e.g. proteins or polysaccharides, through the membrane, thus being able to define its characteristics as a mechanical "sieve". The third approach is the morphological, that is, the anatomical study of the glomerular tuft and its microscopical and ultramicroscopical structure. The literature here cited will generally be divided into these three groups.

The most convincing proof that glomerular fluid is a pure ultrafiltrate of plasma has been made possible by direct puncture of the Bowman capsule and withdrawal of fluid for analysis. Richards and his co-workers have developed a method for the collection of glomerular fluid from the amphibian kidney (Wearn and Richards, 1924, 1925). By microanalysis they found that the composition of this fluid in general corresponded

to that of serum. It differed only in the respect that it was protein-free as far as it could be detected by the analytical method used. These observations have principally been confirmed by Ekehorn (1931) in the frog, and by Walker, Bott, Oliver and MacDowell (1941) in the rat and guinea pig.

Through the introduction of the concept "renal clearance" by Möller, McIntosch and Van Slyke in 1929 a great step forward in the study of renal physiology was made possible. Möller and his co-workers investigated the excretion of urea and its dependence on the plasma concentration and were thus able to calculate the excretory capacity of the kidney for this substance.

The "renal clearance" of a substance can be defined as the amount of plasma that is "cleared" of its content of the substance in question per unit of time. This plasma volume is actually a virtual one because only a fraction of the plasma content of the substance is removed by the kidney at a single passage of blood through the renal capillaries, but still this measure of the renal excretory capacity has been of the greatest value in renal physiology.

The study of the excretion of creatinine, initiated by Rehberg (1926) and followed by a long series of investigations by different authors, showed that the clearance of this substance gave a measure of the glomerular filtration rate of water in the dog and rabbit and some other animals. Unfortunately this was not so with humans because it was shown by several authors that here there exists an active tubular excretion of creatinine in addition to the glomerular filtration.

Working independently Smith and Richards and co-workers found that inulin, a fructose polysaccharide, had all the characteristics that made it suitable as a test substance for the estimation of the glomerular filtration rate. In addition to the indirect evidence of inulin's suitability for this purpose advanced by these authors, Hendrix, Westfall and Richards (1936) demonstrated in *Necturi* and frogs that the concentration of inulin in the glomerular fluid was equal to that of plasma, that is, in spite of being a relatively large molecule it passes through the glomerular filter as easily as water. Many other substances have been tested, for example, mannitol and sodium thiosulphate, but inulin clearance has proved to be the best means to study water transport through the glomerular membrane.

While some criticism has been raised against inulin (Ferguson and others, 1950; Eggleton and Habib, 1951), the recent investigation of Kennedy and others (1953) confirms the position of inulin.

The driving forces concerned in the formation of glomerular fluid have also been the subject of many investigations. The filtration pressure is determined by the glomerular capillary blood pressure, but this force is opposed by the plasma colloid osmotic pressure, the hydrostatic pressure in Bowman's capsule, and the resistance to flow in the tubules.

By lowering the capillary blood pressure or raising the opposing forces to a point where urine flow ceases it is possible to get an approximate figure for the filtration pressure. Winton (1937) in his review of the physical factors involved in the activities of the kidney summarized his own and the results of other workers and found that the glomerular capillary blood pressure (dog) has a magnitude of 65–75 % of the arterial blood pressure. Selkurt, Hall and Spencer (1949) have demonstrated in dogs that urine flow ceases at an arterial blood pressure of about 60 mm of mercury. Recently Swann (1952) summarized the hydrostatic pressures that are thought to be operating in the kidney:

Glomerular capillary	65—60	mm Hg
Peritubular venules	28—26	,, ,,
Bowman's capsule	28	,, ,,
Interstitial space	25	,, ,,

In the peritubular capillaries the hydrostatic pressure from the heart is almost completely balanced by the interstitial hydrostatic pressure, thus the colloid osmotic pressure of plasma in these capillaries can work almost unopposed when reabsorbing water.

Pappenheimer and his co-workers (1951) in their excellent study on the transport across peripheral capillary membranes found that the permeability of the glomerular membrane to water is about 100 times greater than that of the capillaries of mammalian muscle. The collective pore area available for the passage of water through the glomerular membrane was found to be 1–2 % of its total anatomical surface. In the case of muscular capillaries the area available for free passage decreased for larger molecules such as urea and glucose, and Pappenheimer found a correlation between the magnitude of this area and the molecular dimensions. The interpretation of these findings may be that the dimensions of the larger molecules and those of the "pores" are approaching each other so that there will be a steric hindrance in their passage through the membrane.

Chinard (1951, 1952) has discussed the possible mechanisms of the formation of the glomerular fluid. From thermodynamic considerations he derives, without making any assumption as to the mechanism of passage, an expression for the glomerular clearance of any test substance. By analysis he found that this passage has the nature of a diffusion of the molecules through the membrane rather than a passage in bulk by hydrodynamic filtration. The rate of net passage is proportional to the diffusion coefficient of the substance in the capillary wall. Therefore, he concludes, all substances including proteins cross the membrane at a finite rate. The diffusion coefficient in the vessel wall of a low-molecular substance may be close to its diffusion coefficient in water, while the "intramural" coefficient of a large molecule, e.g. a protein, may be of

such a low order of magnitude as to be negligible. In contrast to Pappenheimer and co-workers, Chinard considers the glomerular capillary wall to be a gel-like structure made of fibers of molecular dimensions, the entire surface of which is permeable to water, rather than impermeable plates with discrete pores of relative uniform radius. According to Chinard the area available for free diffusion of water in the human kidneys equals 2.1 m^2, a figure which roughly corresponds to the anatomically calculated total surface of 1.6 m^2 (Vimtrup, 1928).

The second way to elucidate the nature of a membrane as noted above is to study its "sieving" action on macromolecules.

Conclusive evidence of such action was reported by Bayliss, Kerridge and Russell (1933). They demonstrated that after injection of different proteins into the blood of cats and rabbits only those proteins with a molecular weight equal to or lower than that of hemoglobin—for example, gelatin, Bence Jones protein and egg albumin—could pass into the urine. Hemoglobin (mol. wt. 68,000) was excreted when the plasma level exceeded a certain limit. Serum albumin or globulin, edestin, casein and hemocyanin, all of which have higher molecular weights than hemoglobin, were not excreted.

The excretion of hemoglobin has been discussed in a number of publications. Monke and Yuile (1940) measured the hemoglobin/creatinine clearance ratio and found that this quotient averaged 0.03 when the serum concentration of hemoglobin exceeded a level of about 100 mg per 100 ml. Below this critical level no hemoglobin appeared in the urine. This indicates a tubular reabsorption of hemoglobin which has a limited capacity, as they found that its maximal rate in a medium-sized dog was about 2 mg per minute. They conclude that about 3% of the glomerular pores were permeable to this protein. In humans McDonald, Miller and Roach (1951) found that the glomerular permeability to hemoglobin averaged 13% of that to inulin. In similar experiments Yuile and Clark (1941) calculated that about 75% of the glomerular pores were permeable to myoglobin (mol. wt. 17,500). Brandt, Frank and Lichtman (1950) estimated the hemoglobin/inulin clearance ratio in normal and proteinuric subjects but were not able to demonstrate any increase of this ratio in the proteinuric cases. They conclude that the glomerular permeability in terms of "pores" is not the factor determining the degree of proteinuria.

The observations of Bayliss (1933) were confirmed in dogs by Marshall and Deutsch (1950) using conalbumin (mol. wt. 87,000), ovalbumin (44,000), betalactoglobulin (35,000), ovomucoid (27,000), and lysozyme (17,200). The first of these substances having a molecule larger than that of hemoglobin was not able to pass through the renal filter while the others were excreted into the urine. The authors conclude that besides the molecular weight, the shape of the molecules may also influence passage through the glomerular membrane. Addis and others (1951) working with rats reached the same conclusion.

Bing and Harboe (1952) made similar observations but found that fetuin, a protein component from the serum of newborn calves (Pedersen, 1947) which has a lower molecular weight than hemoglobin, did not appear in the urine of calves or rabbits.

Hoffman and Kozoll (1946) investigated the behavior of gelatin in this connection. They studied the clearance in human subjects of three different gelatin preparations with different average molecular weights and found that the gelatin creatinine clearance ratio was higher for the low molecular preparation (37,000) than for the medium and high molecular preparations (47,000 and 58,000).

The ability of the diseased kidney to excrete protein has been the subject of much study. Many theories have been developed in attempts to explain this common symptom of kidney damage. However, no convincing evidence has been put forward to indicate a tubular secretion of protein. Most authors agree that the urinary proteins have a glomerular origin and that they "leak" in some way into the primary urine through the glomerular membrane. Terry and co-workers (1948) demonstrated that when dog plasma is injected repeatedly into dogs there will be an increase in plasma protein concentration, and at a plasma concentration of about 10 g per 100 ml they found that protein appeared in the urine. When the plasma protein concentration is allowed to fall the proteinuria vanishes, and histological controls show no damage to the kidney. The authors claim that their experiments establish a renal threshold for plasma protein, that is, a mechanism similar to that of hemoglobin excretion.

No absolute proof that the capsular fluid is entirely protein free has been published. Wearn and Richards (1924) in their capsular puncture experiments were only able to prove that primary urine did hold less than about 20 mg of protein per 100 ml because of the lack of sensitivity in the methods used. Ekehorn (1931) stressed that most of his investigations showed the presence of slight traces of protein in the capsular fluid. Assuming a leakage of the glomerular membrane that will permit an amount of about 10 mg per 100 ml to pass the filter, and assuming that no protein is absorbed during the passage through the tubules, the result will be a proteinuria of about 10 g per 24 hours. This raises the question of whether or not albuminuria might represent an increased glomerular permeability or decreased tubular reabsorption combined with a constant leakage of protein through the glomerulus.

Numerous studies have clearly demonstrated the ability of the tubules to absorb proteins, a process commonly known as "athrocytosis". The reabsorption of hemoglobin has been referred to above. Smetana (1947) showed the tubular reabsorption of proteins labelled with diazotized dyes in rats, mice and dogs. Dock (1942) and Gilson (1950) injected Evans blue into rats and studied the absorption of the albumin-dye complex in the cells of the proximal convoluted tubules. Gilligan,

Altschule and Katersky (1941) found that after injection of hemoglobin in normal humans there may be a transient proteinuria besides the urinary hemoglobin which might be interpreted as a result of an overload of the protein reabsorptive capacity of the tubules by hemoglobin.

Bing (1933, 1936) demonstrated that the proteinuria varies proportionally with the glomerular filtration rate. He considers the histological feature of the tubular epithelium in the nephrotic syndrome to be a picture of the reabsorption of urinary proteins which have leaked out through the damaged glomerular membrane. This interpretation is generally accepted today.

Squire (1953) has recently discussed the clearance of serum albumin in different kidney diseases. He concludes that the capacity of the tubules to reabsorb protein is uninfluenced by the nature of the protein presented to them. The proteins seem to compete with each other on equal terms. If the glomerular membrane is to be regarded as a sieve, he finds that an enlargement of the "holes" may explain the presence of protein in the urine.

Thus far experiments have been referred to in which various proteins have been used as the test substance. However, other substances have been used as well. Accordingly, Shaffer, Critchfield and Carpenter (1948) injected polyethylene glycols of different molecular weights into dogs and found that preparations with a molecular weight of 4,000 or less had the same clearance as creatinine. A preparation with a slightly higher molecular weight (6,000) had a clearance that was 60–70% of that of creatinine.

Beattie and Corcoran (1949, 1952) investigated the renal clearance of levan, a fructosan isolated from Italian rye grass, and of irisin from Iris rhizomes, both of which have molecular weights somewhat higher than that of inulin. In dogs both substances gave a polysaccharide/creatinine clearance ratio of less than unity and the quotient decreased in successive clearance periods apparently because of the polydispersity of the material and the more rapid excretion of the more filterable low molecular components. In humans, however, the ratio of polysaccharide clearance to that of mannitol averaged 1.1 which here demonstrates that levan was freely filterable without any restriction in passing the membrane. This indicates that the normal human glomerulus is apparently a less retentive filter than that of dogs.

In experiments in patients with hypertensive disease or showing proteinuria, the levan clearance was decreased relatively to mannitol clearance which these authors interpret as a result of a decreased glomerular porosity. The more protein found in the urine the lower was this ratio.

Beattie and Bell (1953) made a series of experiments using irisin and two preparations of bacterial levan. In these tests a similar decline in

the renal clearance of the polysaccharides was found indicating a non-homogeneity of the preparations used.

Ingelman and Halling (1949) have studied the physico-chemical properties of dextran isolated from the urine of surgical patients who had received dextran infusions to prevent or treat shock. The average molecular weight of the most high-molecular fraction of this urinary dextran was estimated by ultracentrifugation and was found to be about 36,000. The more high-molecular fractions in the solution given were thus found to be retained by the glomerular filter.

Grotte, Knutson and Bollman (1951) investigated in rats the urinary excretion of dextran after injection of different dextran fractions with molecular weight averages varying between 24,000 and 205,000. Their results show the presence of dextran in the urine in all experiments with the amounts excreted being inversely correlated to the average molecular weight of the preparation injected. The molecular distribution of the dextran fractions given and of the urinary dextran was not investigated in these experiments.

Friberg and co-workers (1951) made similar experiments on rabbits and found that fractions with an average molecular weight of 40.000 or less were excreted through the kidney while more high-molecular fractions were not detected in the urine.

Brewer (1951) estimated in rabbits the clearance of three dextran fractions with average molecular weights of 38,000, 25,000, and 5,000–10,000 respectively. He compared these results with simultaneously determined creatinine clearance and found the clearance of the different fractions to be 6, 19 and 100% of the glomerular filtration rate. He concluded that dextran is mainly excreted by glomerular filtration and if any tubular excretion or reabsorption occurs it must be relatively small. He discussed the existence of a distribution of pore sizes in the glomerular membrane, all the pores being permeable to molecules of weight about 7,000, 20% of the pores being permeable to molecules of 25,000 and 6% at 38,000.

Grönwall and others (1952) have studied the renal excretion of dextran in human subjects and found that the most high-molecular fraction excreted into the urine had an intrinsic viscosity of 0.20, which corresponds to a molecular weight of about 45,000. They point out that this limit might be different for other types of dextran having different degrees of branching of the molecules and they show that by knowing the distribution of the different sizes of dextran in the solution infused it is possible to predict with a fair degree of accuracy the amount of dextran that will be excreted through the kidneys.

Campbell and co-workers (1953) have recently studied the effect of molecular weight on the renal excretion of polyvinyl pyrrolidone, a plasma substitute, in humans. They found that the rate of passage of these molecules through the kidney is inversely related to the molecular

weight and that even very large molecules with molecular weights in excess of 100,000 are found in the urine in small amounts.

Finally, the morphological study of the functioning units in the kidney parenchyma has revealed significant contributions. Since Malphigi in 1666 first described the glomerular corpuscle many studies of the discrete anatomy of the functioning units of the kidney, the nephrons, have been published. The more important ones will be noted here. Bowman (1842) rediscovered and described the glomeruli suggesting in part the modern theory of function when he said: "It is difficult to conceive the disposition of parts more calculated to favour the escape of water from the blood than that of the Malphigian body."

Vimtrup (1928) investigated the number, shape, structure and surface area of the glomeruli in the kidney of man and some mammals. In man the number averaged about 1,000,000 in each kidney, their total volume was calculated to be 4.2 cm^3 and total surface area of the capillary network about 0.78 m^2.

The morphology of the membranes covering the glomerular tuft has been studied exhaustively by Zimmerman (1933) and McManus (1948). The glomerular capillary wall consists of three primary membranes: (1) the capillary endothelium, usually considered to coat the inside of the capillaries as a continuous layer having no openings or defects that are visible by ordinary microscopy; (2) the basement membrane, a "hyaline" or thin membrane composed of proteins and polysaccharides; (3) the capsular epithelium, that is, the visceral layer of Bowman's capsule composed of low, squamous epithelial cells, and considered by some authors to be a discontinuous layer. By means of a special technique Zimmermann (1933) demonstrated that these epithelial cells have a characteristic crab-like appearance exhibiting delicate, discrete irregular processes investing the basal membrane as a web-like pericytium.

With electron microscopy Pease and Baker (1950), Dalton (1951), and Oberling, Gautier and Bernhard (1951) have conclusively demonstrated the detailed morphology of these pericytial structures. The form and arrangement of these lattice-like net-works of interdigitating processes covering the basement membrane are suggestive of mechanical analogies where fins and ribs are used to strengthen and support tubular systems against bursting pressures. In some instances, Gautier and co-workers (1950) observed a very thin membrane between these ribs which presented a very fine regular structure resembling a honeycomb with a frequency of the structural details of a magnitude of 500 to 1,000 Å. In cross sections the basement membrane was found by Dalton (1951) to be 0.1 μ in thickness.

The process of urine formation, as can be seen from this brief review, is relatively well documented in many aspects while no more than scattered data are available concerning some important details in the

function of the nephrons. The process of glomerular filtration and the results of this process, the fully elaborated urine and its composition, is generally well understood, but the data on the filter *per se* are relatively scarce and incomplete. The discussion concerning the detailed physical structure of the filtering membrane is far from settled. Similarly the question of proteinuria has been solved to some extent, but whether or not there are in different diseases different mechanisms for this condition has only been briefly considered in the literature.

CHAPTER 2

The Problem, and General Remarks on the Methods Used

When studying the influence of dextran injections on the edema of patients showing the nephrotic syndrome (Wallenius, 1950) the author made some observations regarding the excretion of this substance via the kidneys. Assuming that a polymolecular colloidal substance such as dextran could in some way be applied as a gauge to determine the state of permeability or porosity of the glomerular capillary membranes and thus be able to clarify the nature of different types of proteinuria, some preliminary investigations were made (Wallenius, 1952) which showed the applicability of this substance to this particular problem. In the present work some experiments in humans and animals will be reported with particular reference to the mechanism for the passage of a polymolecular dextran preparation through the kidneys into the urine. Also, some of the methods that had to be developed and adapted to this problem in order to determine dextran quantitatively as well as qualitatively in blood and urine will be discussed.

The principle of the method used was as follows. A macromolecular compound, dextran, with a molecular size distribution within a selected range was filtrated through the glomerular capillary wall. The filtrate was then collected and analyzed for dextran observing particulary the occurrence of the different sizes of the molecules. Consequently the composition of the filtrate, in this case urine, will indicate the porosity or the "permittivity" of the membrane to the substance selected, and by comparing the composition of the solution on both sides of the filtering membrane quantitative aspects of the filtrability of the different molecular sizes can be obtained.

CHAPTER 3

Methods

Character of the Dextran Preparations Used

Some data on the chemical nature of dextran with special regard to the dextran preparations used in these experiments will here be given. For a more comprehensive description of the general nature of dextran reference is made to Grönwall and Ingelman (1944, 1945), Evans and Hibbert (1946), and Ingelman and Halling (1949).

Dextran is a polysaccharide that is synthesized by the bacteria *Leuconostoc mesenteroides* and some other bacterial strains when grown in a medium containing saccharose. Chemically it is composed solely of glucose molecules that are linked together in branched chains by 1:6- and 1:4-glucosidic linkages, which first-mentioned linkages constitute the main chain. The degree of branching varies in dextrans from different bacterial strains. The native dextran obtained from a *Leuconostoc* culture has a very high molecular weight amounting to several millions. By treatment with acid and heat or by other means these molecules can be broken down to their monomers, but by interrupting the hydrolytic process at an earlier stage, molecules of any order of magnitude can be obtained.

The dextran substance used in these experiments was placed at the author's disposal by Pharmacia Ltd., Uppsala. It is identical with this company's commercial plasma volume expander "Macrodex".[1] The native dextran was obtained from *Leuconostoc* strain B-512 and was degraded through acid hydrolysis and refined according to the methods of Grönwall and Ingelman (1944, 1945). The degree of branching was about 15–20 1:6-linkages for each 1:4-linkage (Granath, 1953).

In order to obtain as homogenous fractions as possible for calibration purposes the substance was fractionated from a water solution by precipitation with ethyl alcohol into a series of subfractions following the procedure of Ingelman and Halling (1949). The fractions so obtained were refractionated from a 1% solution into subfractions which were further reprecipitated in order to minimize the effects of coprecipitation. The final fractions were isolated and dried at + 100° C for about 24 hours and kept in an desiccator over silica gel. The solutions of the pure substances

[1] Some preparations with varying molecular weight data were specially prepared and kindly made available by dr. B. Ingelman, Ph. D., Uppsala.

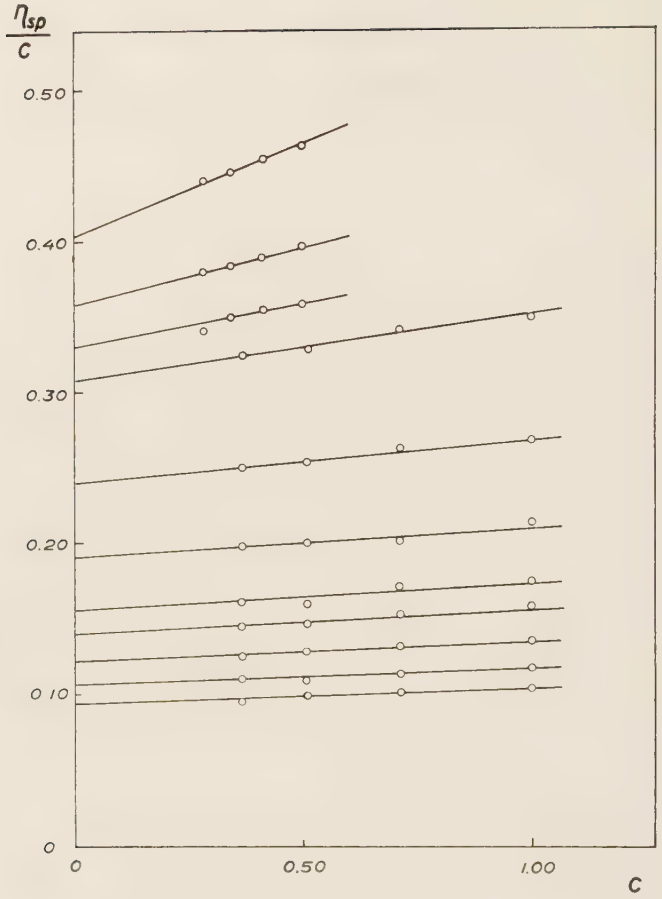

Fig. 1. Viscosity data for narrow dextran fractions used in the calibration experiments.

were prepared by weighing and dissolving in distilled water under heating to +100° C, and after cooling filled to volume with solvent. In this way a series of eleven narrow fractions scattered over an adequate molecular weight range were approved and used in the calibration experiments described on page 29.

The specific viscosity, η_{sp}, of each fraction was estimated at +20° C with an Ostwald capillary viscometer (efflux time for water 70 sec.) at four different concentrations with water as solvent. The intrinsic viscosity (= limiting viscosity number) $[\eta]_{20°}$ of the fractions was calculated according to the formula:

$$[\eta]_{20°} = \lim_{c=0} \eta_{sp}/c \qquad (1)$$

Table 1. *Viscosity data for dextran fractions.* η_{sp} = *specific viscosity, c = dextran concentration in g per 100 ml.*

Fraction No.	η_{sp}/c				
	c = 1.00 %	c = 0.714 %	c = 0.510 %	c = 0.364 %	c = 0 %
1	0.104	0.101	0.099	0.095	0.094
2	0.118	0.114	0.109	0.110	0.106
3	0.136	0.132	0.129	0.125	0.121
4	0.159	0.153	0.147	0.145	0.140
5	0.175	0.171	0.160	0.161	0.155
6	0.214	0.199	0.200	0.198	0.190
7	0.264	0.255	0.251	0.248	0.237
8	0.350	0.342	0.329	0.324	0.307
	c = 0.500 %	c = 0.417 %	c = 0.347 %	c = 0.289 %	c = 0 %
9	0.359	0.355	0.350	0.340	0.330
10	0.397	0.389	0.384	0.380	0.358
11	0.463	0.455	0.446	0.440	0.404

where $\eta_{sp} = (\eta_{rel} - 1)$ and η_{rel} equals the viscosity of the solution relative to that of water and c denotes the polymer concentration in grams per 100 ml. Correction for the density of the solutions was applied but the kinetic energy corrections were assumed to be negligible. The results appear from Fig. 1 and Table 1.

In Fig. 2 is given the correlation between intrinsic viscosity and molecular weight for dextran of this type as calculated from ultracentrifuge and diffusion measurements.[1]

A general relationship between intrinsic viscosity and molecular weight is given by the function (Mark, 1938):

$$[\eta] = K \cdot M^a \qquad (2)$$

where K and a are constants, typical for the solute-solvent system used. The experimental data in Fig. 2 satisfy rather well an equation of this type, and by the method of least squares K is found to be 0.72×10^{-3} and a 0.53.[2] In this connection it is of interest to note that Hulburt,

[1] These experiments were carried out by Tekn. lic. Kirsti Granath, Institute of Physical Chemistry, University of Uppsala, who has very kindly made the data available to the author.

[2] Wales, Marshall and Weissberg (1953) have recently investigated this relationship for dextran (B-512) at 25°C, and they state that the approximative values of $K = 10^{-3}$ $a = 0.5$ correspond rather well to the experimental results within the molecular weight range 20,000 to 250,000.

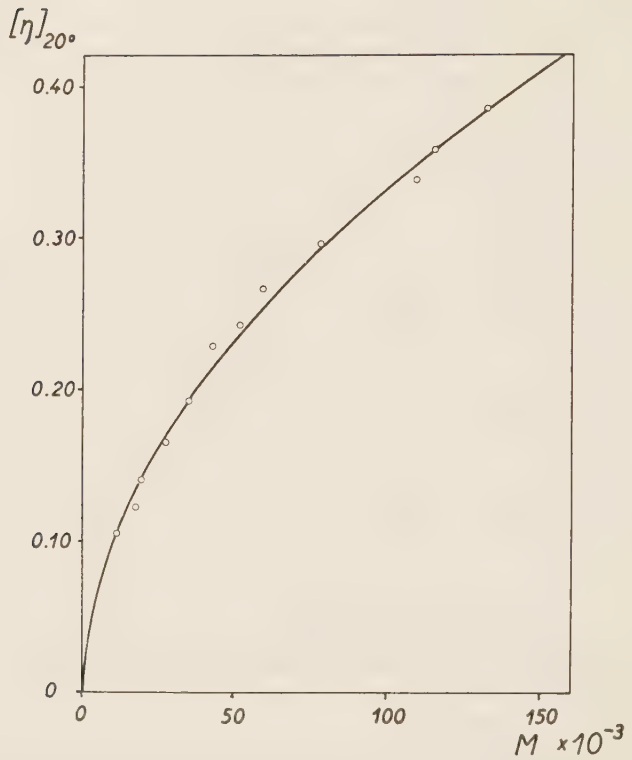

Fig. 2. Relationship between intrinsic viscosity $[\eta]$ and molecular weight, calculated from sedimentation and diffusion measurements, for partially degraded dextran (B 512).

Harman, Tobolsky and Eyring (1943) deduced theoretically that a will assume a value of $\frac{1}{2}$ for a flexible, randomly kinked polymer chain, i.e. matted coil molecules which during flow move together with the immobilized solvent, while for a completely rigid, rod-shaped molecule, the theoretical value of a will be 2. Free-draining chains where the solvent can flow freely along and around the various parts of the molecule should have a value of a that is near 1 (Huggins, 1942).

The relationship between diffusion coefficient and molecular weight for this type of dextran is given in Fig. 3. The diffusion coefficients, D_A, refer to measurements made at 20° C with water as solvent and are calculated according to the area method of Lamm (1937). In order to get an impression of the dimensional magnitudes of the different molecular sizes their effective diffusion radius, that is, the radius of a sphere with equivalent diffusion coefficient, was calculated according to the formulas of Stokes and Einstein (1905):

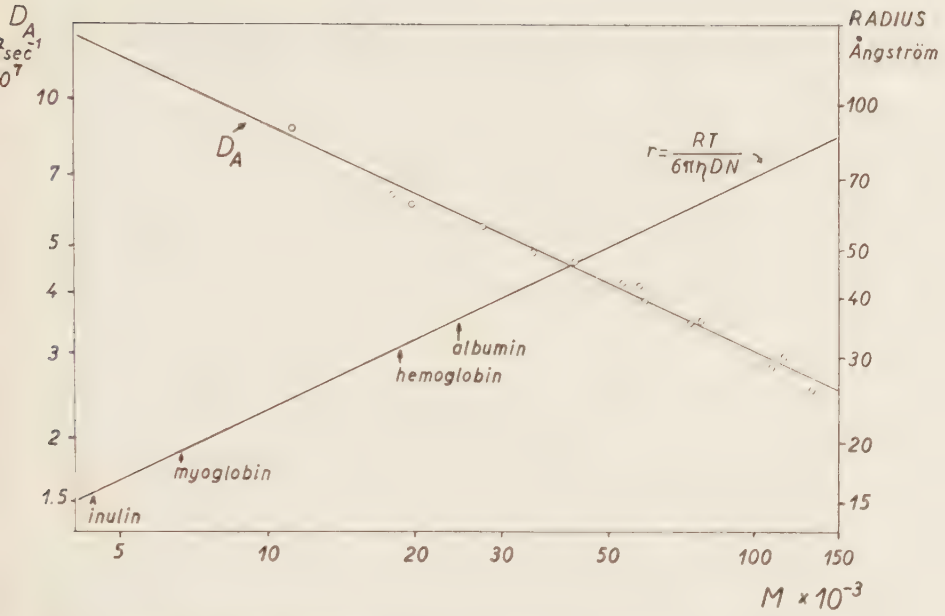

Fig. 3. Relation between molecular weight M, diffusion coefficient D_A, and effective diffusion radius (Einstein-Stokes) r, for partially degraded dextran (B 512). The diffusion radius for inulin, myoglobin, hemoglobin and albumin are indicated by arrows.

$$r = \frac{RT}{6\pi\eta DN} \qquad (3)$$

where R is the gas constant, T absolute temperature, η the viscosity of water, D the diffusion coefficient, and N Avogadro's number. The magnitude of the molecular radius r is given in Ångström units. For comparison the corresponding radius for serum albumin, hemoglobin, myoglobin, and inulin are indicated with arrows (the data are quoted from Pappenheimer, 1951).

Quantitative Determination of Dextran in Blood and Urine

Several different methods for the quantitative analysis of biological fluids for dextran have been described (Klevas, 1944; Hint and Thorsén, 1947; Bloom and Willcox, 1951; Sällman, 1952; Jacobsson and Hansen, 1952; Metcalf and Rousselot, 1952). The author has tested several of

these methods for applicability to his own experiments. A modification of the anthrone method proved to be the best for his purpose. Since a detailed description of this method has recently been published (Wallenius, 1953), only a summary will be presented here.

Principle of the method

After treatment with yeast to remove glucose and deproteinization the test solution is heated together with an anthrone-sulphuric acid mixture which then turns blue or blue green. The intensity of this color is within certain limits proportional to the dextran concentration.

Reagents

(a) Anthrone solution. 2 g anthrone (9-10-dihydro-9-oxoanthracene) is put in a 1000 ml volumetric flask and 300 ml of distilled water is added. The flask is then slowly filled to volume with concentrated sulphuric acid.

(b) Zinc sulphate solution, 10%.

(c) Sodium hydroxide solution, 0.5 normal.

(d) Washed yeast. Baker's yeast is washed 3 times with normal saline. Then after decanting the heavy suspension is transferred to a thick pad of filter paper and allowed to drain until practically all the moisture is removed.

(e) Dextran standard solutions of suitable concentrations (20, 50 100 mg per 100 ml) prepared from oven-dried (100° C for 24 hours) dextran substance.

Technique

Serum.—A pea-sized lump of washed yeast is placed in 1.5–2 ml of serum.

After stirring the mixture is incubated at 37° C for 1 hour. It is then centrifuged and 1.0 ml of the serum is transferred to a small flask. Add 7 ml of distilled water, mix, add 1 ml sodium hydroxide, mix again. Centrifuge after 10 minutes but do not filter as the slightest contamination with cellulose fibers gives erroneously high readings. Treat the supernatant clear solution with anthrone as described below. If the dextran concentration in the serum is high (more than 150 mg per 100 ml) an aliquot of the supernatant fluid is diluted with distilled water so that 1 ml of the dilute fluid contains about 50-100 micrograms per ml.

Transfer 1 ml of this solution to a large pyrex test tube. Add 10 ml of the anthrone reagent and mix. Place the tube in a boiling water bath for exactly 10 minutes and cool immediately afterwards in cold running water.

The optical density of the blue solution is read within 30 minutes

(1 cm cuvette depth, wavelength 620 mμ) against a water blank which has been prepared exactly as the test solution. The same procedure is simultaneously applied to the standard solutions and a standard curve is plotted.

Urine.—Concentrated urine specimens usually yield a high non-dextran blank value. These non-specific anthrone-reacting substances are mostly removable by dialysis through a cellophane membrane (Wallenius, 1953) which, however, must be impermeable to dextran molecules and tested accordingly. After dialysis the procedure is the same as for serum.

In most clearance experiments the urine is so diluted by a suitable water load and the dextran concentration of the urine is so high that these precautions may be omitted as the non-specific blank error becomes negligible.

Error of the method

The standard error of this modification of the anthrone method has been calculated from double determinations of 30 serum samples with a dextran concentration range between 20 and 100 mg per 100 ml. A Beckman spectrophotometer, model B, was used in all experiments. The standard error σ was found to be $\pm$ 1.8% calculated according to the formula:

$$\sigma = \pm \sqrt{\frac{\sum d^2}{2N}} \qquad (4)$$

(Dahlberg, 1948) where d is the difference between the double determinations in % and N the number of specimens.

Isolation of Dextran from Blood and Urine

Isolation from blood

One ml of serum or plasma was mixed with 4 ml of a saturated picric acid solution in a centrifuge tube, and centrifuged for 10 minutes. Two ml of the clear supernatant was then transferred to another centrifuge tube and 8 ml of 99.5% ethanol was added (final ethanol concentration 80% v/v). After thorough mixing the tube was then placed in a refrigerator (+ 2° C) for 60 minutes and then carefully centrifuged at 10,000 r.p.m. for 15 min. The deposit was then washed twice with cold ethanol, centrifuged, decanted and then dissolved in 5 ml distilled water. The concentration of dextran in this solution was determined with the

Table 2. *Recovery of dextran from serum and urine after deproteinization with picric acid and precipitation of dextran at 80 % alcohol concentration.*

Concentration in mg per 100 ml		Recovery, %	Concentration in mg per 100 ml		Recovery, %
Serum	Isolate		Urine	Isolate	
87.2	86.3	99.2	370	363	98.2
183	176	96.5	1750	1730	99.0
170	164	96.8	1910	2060	107.6
131	133	101.5	847	857	101.0
278	288	103.5	3620	3700	102.0
169	175	102.4	880	865	98.5
201	195	97.0	1360	1400	103.0
64.2	67.0	103.5	3450	3520	102.0
37.8	38.7	102.3	666	660	99.0
332	340	102.3	231	235	101.4
Average recovery: 100.5					101.2

anthrone method and a final dilution with distilled water was made to a dextran concentration of 0.5 mg dextran per 100 ml.

Isolation from urine

The urine specimen was diluted 1:10 with distilled water and then the same procedure as for blood was applied.

When treating undiluted urine samples with ethanol some non-dextran material will sometimes fall out but this non-specific precipitation can be avoided by a tenfold dilution. Furthermore as all clearance experiments were performed after a sufficient hydration of the subject, the urine when voided was quite dilute. However, to test the sufficiency of this precaution, 10 urine samples chosen at random from non-hydrated subjects were treated as above with dilution and precipitation with picric acid and alcohol. When reading the turbidity of the alcohol mixture after sufficient time in the refrigerator and remixing no detectable turbidity was found.

Recovery

In order to test the recovery of dextran after precipitation with 80 % ethanol some experiments were performed, the results of which are shown in Table 2.

In another series of experiments where dextran was added to serum and urine and thereafter isolated as above it was established by turbidity titration experiments that the treatment with picric acid

and alcohol involved no detectable change in the molecular weight distribution of the dextran used (see page 44). Some other procedures for deproteinization, with trichloroacetic acid, for example, had to be abandoned because of such a change.

Determination of Molecular Weight Distribution of Dextran

Dextran is a polymolecular substance, that is, it is composed of a series of different molecular sizes. By various methods it is possible to get a measure of the average molecular weight of the substance. However, this characterization is often not sufficient, and for more precise knowledge a measure of the actual distribution of the different molecular sizes is necessary. This is most often done by fractionation of the substance into a series of relatively homogeneous subfractions, usually by precipitation with a non-solvent, and then estimating the weight and the average molecular weight of each subfraction.

However, when working with the physiological experiments discussed in this paper it was found that such a procedure as that outlined above was not applicable. The amounts of dextran that could be isolated and recovered from serum and urine were too small to be analyzed in that way.

A more suitable principle was that devised by Morey and Tamblyn (1945) for the analysis of cellulose acetate-butyrate and which has more recently been used on different polymers by Oth (1949), Harris and Miller (1951), Bischoff and Desreux (1951) and Mussa Ivaldi Vercelli (1951). With some modification of this principle a method has been developed which enables the measurement of the molecular weight distribution of partially degraded dextran preparations using only 0.01 mg of the substance, an amount that can easily be recovered from e.g. blood samples in the physiological experiments. The method requires a somewhat complicated and detailed calibration procedure, but when this is done, a measurement of an unknown sample of dextran is fairly rapidly obtained.

Principle

When adding a non-solvent (ethanol) to a dilute solution of dextran, the larger molecules which are less soluble precipitate first, followed by the smaller molecules as more alcohol is added. The increment of dextran that precipitates at each increment of the precipitant added is measured by turbidimetric means. Thus, a kind of precipitation curve is obtained and after properly verified corrections are made, this curve can be transformed into a molecular weight distribution curve.

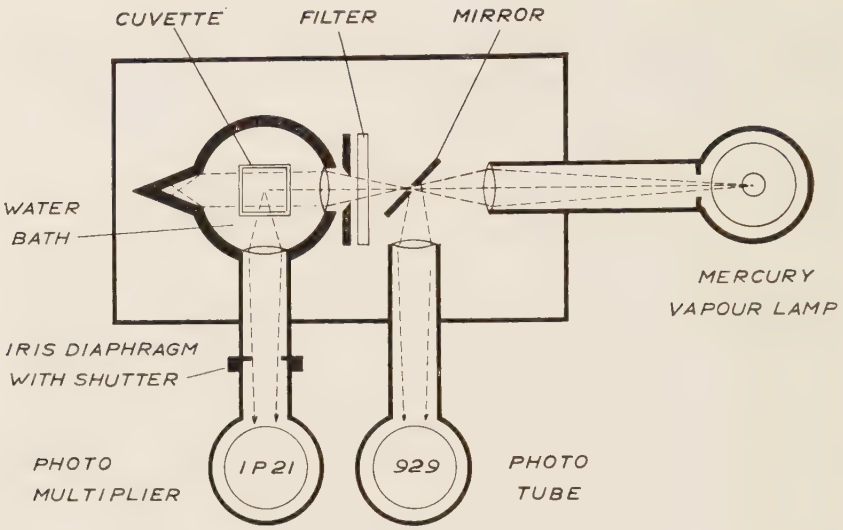

Fig. 4. Schematic diagram of the turbidimeter.

Apparatus

The turbidimeter[1] used in these experiments is based on the light scattering instrument of Stein and Doty (1946) and is connected with a photocell unit constructed similar to that of Hadow, Sheffer and Hyde (1949).

The principle of the optical part of the apparatus is shown in Fig. 4. A square glass cell (internal measurements 20 × 17 × 27 mm) is immersed in a water bath kept at constant temperature (20° ± 0°1 C). The light from an air-cooled mercury vapour discharge lamp (Luma special, 120 w) is directed through the cell via an optical system making the resulting beam essentially parallel, and a monochromatic filter (Zeiss C, maximum transmittance at 4358 Å) is used. After having passed the cell, the light is trapped by a black conical recess in the water bath.

The scattered light from the turbid solution in the cell is picked up transversely to the incident beam by a lens which directs the light to a multiplier phototube (1 P 21). The peripheral part of the incident light beam is reflected into a second phototube (929) by means of an annular metal mirror. To minimize fluctuations in light intensity of the mercury lamp, it is fed through a constant voltage transformer. Fluctuations

[1] The author prefers to use the term *turbidimetry* for the measurement of the light scattered by a turbid suspension transversely to the incident beam. Some authors use the term *nephelometry* for this type of measurement, and *turbidimetry* for the measurement of light transmitted in a parallel direction through the turbid medium (cf. Drabkin, 1944).

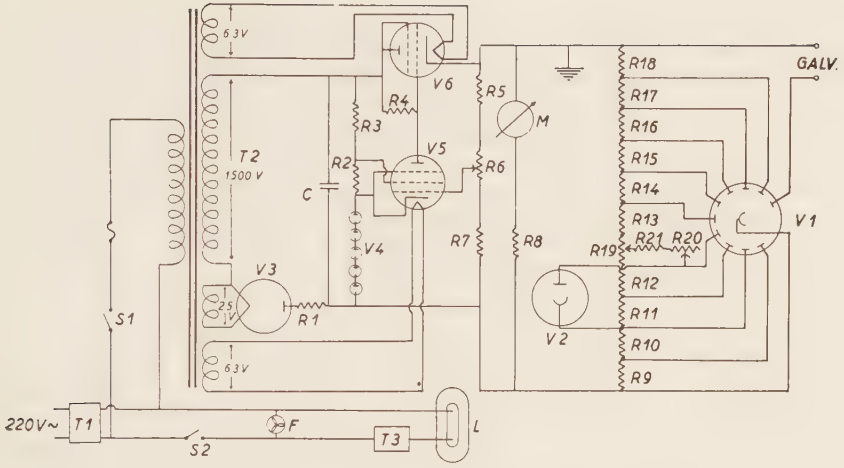

Fig. 5. Circuit diagram of the electronic part of the turbidimeter.

Explanation of symbols:

V 1	1 P 21 Multiplier phototube.	R 1	50 k ohms, 10 w.
V 2	929 Phototube.	R 2	250 k ohms, 1 w.
V 3	2 × 2 Rectifier.	R 3	1 megohm, 2 w.
V 4	5–991 voltage regulators.	R 4	2 megohms.
V 5	6SJ7.	R 5	3 megohms.
V 6	6V6.	R 6	100 k ohms potentiometer.
L	Mercury vapor discharge lamp 120 w (Luma Special).	R 7	1 megohm.
		R 8	1.5 megohm.
T 1	Constant voltage transformer.	R 9–R 11	100 k ohms.
T 2	4 transformers.	R 12–R 13	50 k ohms.
T 3	Choke for Hg lamp.	R 14–R 17	100 k ohms.
S 1	Main switch.	R 18	50 k ohms.
S 2	Mercury lamp switch.	R 19	100 k ohms potentiometer.
M	Meter, 0–1 mA.	R 20	5 megohms potentiometer.
F	Fan.	R 21	1 megohm.

that still remain are eliminated by the photocell 929, the output of which is used to adjust the amplification of the photomultiplier 1 P 21 mainly according to the principle of Hadow and co-workers (1949) (Fig. 5).

The photomultiplier tube has a regulated high voltage supply principally according to the system of Bereskin (1943) (Fig. 5).

In order to compensate for fatigue and drifting of the photomultiplier tube during use it is necessary to refer repeatedly to a turbidity standard. This consists of a polished plexiglass block of the same dimensions and shape as the glass cell. This standard cell is placed immediately below the glass cell in the waterbath and it can conveniently be brought into the light path, replacing the glass cell, by pulling a lever.

It is very important in this procedure to keep the temperature of the system at a constant value. In order to minimize the influence of the temperature in the surroundings it was decided to work at 20°0 C. The heat of the lamp was kept down by air cooling. Water from a thermostat was pumped through a copper coil near the glass cell and through the double-walled bottom of the waterbath, thus rapidly eliminating the mixing heat which is generated when alcohol is added to the cell contents.

To ensure rapid mixing in the cell the instrument was provided with a motor-driven stirrer with a glass propeller which can be withdrawn from the cell during readings.

The output of the photomultiplier tube is directly measured with a Multiflex galvanometer with a sensitivity of 2×10^{-9} amps. per mm of the scale. In order to maintain stability the maximum output was always kept below 1.5×10^{-6} amps. (cf. Engstrom, 1947, and Kinell, 1953). The galvanometer deflection provoked by the turbidity standard used in these experiments was 9.6×10^{-7} amps. at 110 volts per stage of the photomultiplier tube, which corresponds to a luminous flux of the scattered light of about 10^{-7} lumens. No calibration in absolute turbidity units was made. The galvanometer deflection when the cell contained pure water corresponded to about 2×10^{-7} amps., and the dark current was 4×10^{-9} amps.

Turbidimetric titration

Two ml of a solution containing 0.5 mg dextran per 100 ml water, prepared according to the procedure described above, is placed in the cuvette of the turbidimeter. Thereafter 1.2 ml of anhydrous ethyl alcohol is slowly added from a 5 ml burette (graduated for each 0.02 ml) while the mixture is stirred. After sufficient time for temperature equilibration has elapsed (12 minutes), alcohol is added beginning with 0.10 ml amounts, then increasing to 0.50 ml quantities until a final concentration in the cuvette of 77.8% alcohol (v/v) is achieved. The galvanometer deflection is read 3 minutes after each addition of ethanol. Immediately before each reading the turbidity standard is put into the light beam in order to check the stability of the system. All solutions used were centrifuged or allowed to stand for a sufficient time before use in order to settle dust and other matter that could disturb the turbidimetric measurements.

Determination of the saturation limit law

The procedure described here is mostly quoted from Morey and Tamblyn (1945). However, to fit the dextran-water-ethanol system some modifications have been necessary and accordingly a somewhat detailed description of the calculations required will be given.

The basis of the Morey and Tamblyn method is the so-called "satura-

Table 3. *Viscosity, solubility, and molecular weight data for dextran fractions used in the calibration procedure.*

Fraction	$[\eta]$	C^γ and P^γ when initial conc. in the cuvette is				$f(M)$	$-K$	Mol.w.
		1.00 mg %	0.50 mg %	0.25 mg %	0.125 mg%			
1	0.094	C^γ 0.342	0.165	0.079	0.038	64.00	3.75	9,250
		P^γ 65.80	66.90	68.20	69.25			
2	0.106	C^γ 0.372	0.181	0.087	0.042	61.30	3.63	11,400
		P^γ 62.90	63.80	65.10	66.20			
3	0.121	C^γ 0.398	0.196	0.094	0.045	58.90	3.48	14,500
		P^γ 60.20	61.40	62.40	63.60			
4	0.140	C^γ 0.414	0.203	0.099	0.048	57.20	3.23	19,400
		P^γ 58.55	59.50	60.55	61.60			
5	0.155	C^γ 0.436	0.214	0.105	0.051	55.10	3.10	23,500
		P^γ 56,30	57.25	58.15	59.25			
6	0.190	C^γ 0.476	0.233	0.114	0.056	51.45	3.08	34,700
		P^γ 52.40	53.30	54.40	55.05			
7	0.237	C^γ 0.507	0.248	0.125	0.060	48.60	2.84	52,700
		P^γ 49.30	50.25	51.10	51.90			
8	0.307	C^γ 0.544	0.267	0.132	0.065	45.00	2.64	85,700
		P^γ 45.65	46.55	47.35	48.10			
9	0.330	C^γ 0.551	0.272	0.134	0.066	44.20	2.57	98,800
		P^γ 44.90	45.65	46.40	47.20			
10	0.358	C^γ 0.562	0.277	0.136	0.067	43.30	2.48	115,000
		P^γ 43.85	44.60	45.30	46.20			
11	0.404	C^γ 0.571	0.282	0.139	0.069	42.00	2.42	145,000
		P^γ 42.90	43.65	44.35	45.10			

tion limit law" which relates the solubility limit of a homogeneous fraction of the polymer to its concentration at the point of precipitation and its molecular weight, the temperature being kept constant. The solubility limit is represented by the quantity of nonsolvent that has to be added to cause initial precipitation.

These relationships were studied for dextran by precipitating the series of "sharp" fractions described on page 18, using different starting concentrations of dextran in the cell: 1.00, 0.50, 0.25, and 0.125 mg per 100 ml, and noting the percentage (v/v) of ethanol (P^γ) at which precipitation just starts. The data, that appear from Table 3, were then plotted in a diagram (Fig. 6) and it was found that the resulting line for each fraction in accordance with the theories of Schulz (1937) had the equation

$$P^\gamma = K \log C^\gamma + f(M), \qquad (5)$$

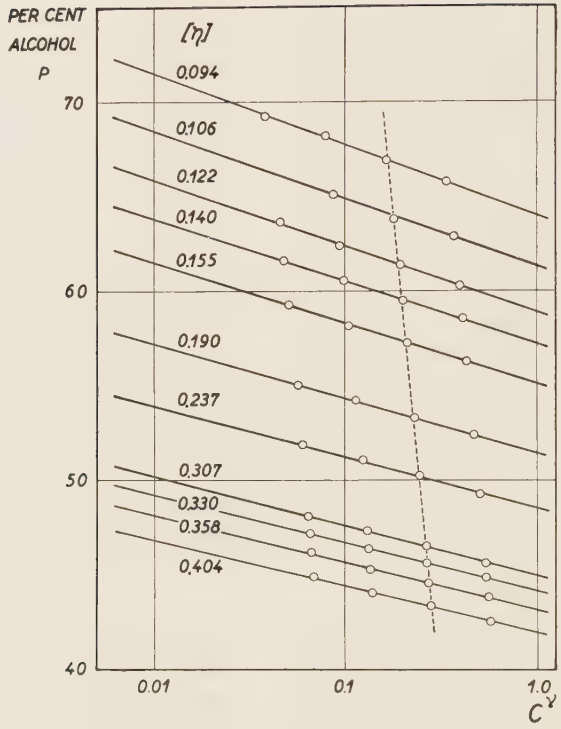

Fig. 6. The relation between dextran concentration C^{γ} (in mg per 100 ml) and alcohol concentration (per cent) at saturation point, for different dextran fractions, denoted by their intrinsic viscosity $[\eta]$.

the "saturation limit law", where C^{γ}, the concentration of dextran in solution at alcohol concentration P^{γ}, is related to the initial dextran concentration (C^{0}) by the "dilution equation"

$$C^{\gamma} = C^{0}(1 - P^{\gamma}/100) \tag{6}$$

and $f(M)$ is a function of the molecular weight.

In equation (5) it is found that

$$P^{\gamma} = f(M) \qquad \text{when} \qquad C^{\gamma} = 1.$$

When the $f(M)$-value for each fraction is plotted against its average molecular weight (Fig. 7) it is found that within the experimental error and within the molecular weight range investigated the relation well fits an equation of the type

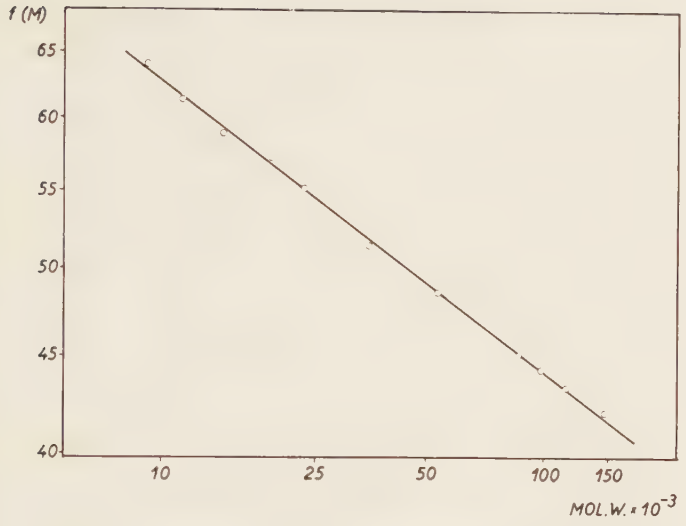

Fig. 7. Logarithmic plot of $f(M)$ as a function of molecular weight.

$$f(M) = k' \frac{1}{M^{a'}}, \tag{7}$$

where k' and a' are constants typical for the precipitation system, in this system found to be: $k' = 2.41$ and $a' = 0.153$ (cf. Schulz and Jirgensons, 1940).

Now, returning to equation (5) and Fig. 6, it is found that the lines for the fractions are not strictly parallel, and when the angle coefficient K of the lines is plotted as a function of $f(M)$ (Fig. 8) a simple linear relationship between these two terms within the range investigated is found. The angle coefficient for this line is by aid of the method of least squares found to be -0.057 and the line is found to pass through the origin. Thus equation (5) can be written:

$$P^{\gamma} = -0.057 f(M) \log C^{\gamma} + f(M). \tag{8}$$

Application of the saturation limit law

Suppose that a dextran preparation with an unknown molecular weight distribution is precipitated by the gradual addition of alcohol, and that by the optical method we can estimate the percentage Z of the polysaccharide that has precipitated out. We can thus draw a graph of the function:

$$Z = G(P) \tag{9}$$

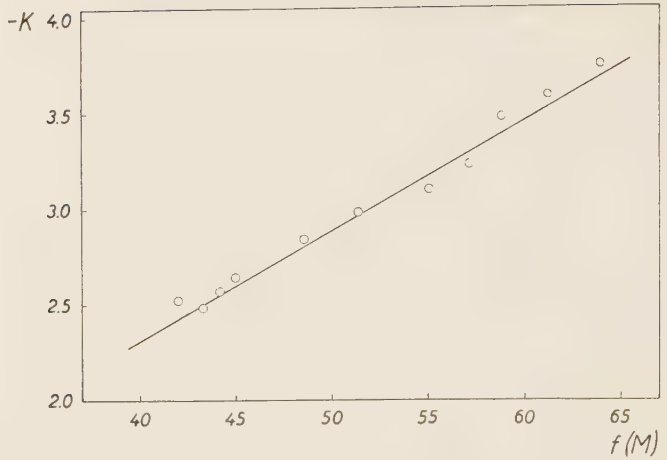

Fig. 8. Relationship between the slope of the lines in Fig. 6 and $f(M)$.

and can read off the corresponding increments of

$$\Delta Z = G'(P)\,\Delta P. \tag{10}$$

The increment ΔZ does not consist of one molecular weight species but contains a distribution around a most probable value. We can, however, assign to each ΔZ some representative value and assume that all of ΔZ is of this single value only.

Now suppose that the total concentration of the polysaccharide is C_T^0 and that it is composed of a series of different molecular weight species with the partial concentrations C_1^0, C_2^0, C_3^0, ... Suppose further that a certain species M_i has such a concentration that the solution is saturated for that species at the concentration P^γ of the precipitant. If this concentration is increased by ΔP, a percentage $(\Delta Z)_i^\gamma$ of the polysaccharide will precipitate. According to the assumption above, all of this $(\Delta Z)_i^\gamma$ is of the species M_i. Then

$$(\Delta Z)_i^\gamma = \delta_i\,(C_i^0/C_T^0)\,100, \tag{11}$$

where δ_i is the fraction of species M_i precipitated in the interval P^γ to $P^\gamma + \Delta P$.

Solving for δ_i we have

$$\delta_i = (C_T^0/C_i^0)\,[(\Delta Z)_i^\gamma]/100. \tag{12}$$

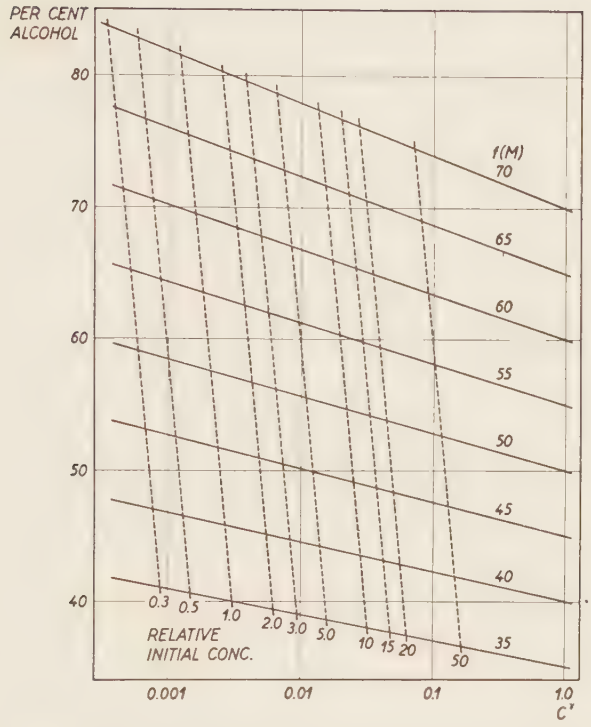

Fig. 9. The relationship between the concentration of precipitant at the precipitation point and the concentration of a molecular species, expressed as a percentage of the total polymer concentration, for different molecular weight species, denoted by their $f(M)$-values.

The concentration of species M_i now remaining in solution at the end of this interval is from equations (6) and (12)

$$C_i^{\gamma+\Delta} = C_i^0 (1-\delta_i)\left(1-\frac{P^\gamma+\Delta P}{100}\right) =$$

$$= \left[C_i^0 - \frac{C_T^0 (\Delta Z)_i^\gamma}{100}\right]\left[1-\frac{P^\gamma+\Delta P}{100}\right]. \qquad (13)$$

At both dilutions P^γ and $P^\gamma+\Delta P$ the solution is saturated with respect to species M_i, and therefore equation (8) is applicable. Rewriting (8) as

$$C_i^\gamma = 10^{P^\gamma/-0.057\cdot f(M)} \times 10^{1/0.057} \qquad (14)$$

and applying equation (14) to the two cases, we have:

$$\frac{C_i^0}{C_T^0}\left(1-\frac{P^\gamma}{100}\right)=\frac{1}{C_T^0}\cdot 10^{P^\gamma/-0.057f(M_i)}\times 10^{1/0.057} \tag{15}$$

$$\left[C_i^0-\frac{C_T^0(\Delta Z)_i^\gamma}{100}\right]\left[1-\frac{P^\gamma+\Delta P}{100}\right]=10^{(P^\gamma+\Delta P)/-0.057f(M_i)}\times 10^{1/0.057} \tag{16}$$

and dividing (15) by (16) we have

$$100\,\frac{C_i^0}{C_T^0}=\frac{(\Delta Z)_i^\gamma}{1-10^{\Delta P/-0.057f(M_i)}\times\left[\dfrac{100-P^\gamma}{100-P^\gamma-\Delta P}\right]}. \tag{17}$$

The ratio C_i^0/C_T^0 is the key to the distribution curve. P^γ and ΔP on the right-hand side of equation (17) are known from the experimental data. As an approximative value for $(\Delta Z)_i^\gamma$ we may, following Morey and Tamblyn (1945), use $(\Delta T)_i^\gamma$, that is, the increment in turbidity at the interval P^γ to $P^\gamma+\Delta P$, expressed as a percentage of the final turbidity. Then only $f(M)$ remains as an unknown. However, by studying $f(M)$ as a function of P for different fractions, as in Fig. 6, it is possible to find an approximate value for this factor.

Along the line $C=1$ in Fig. 9 we thus draw through arbitrarily chosen P values lines according to equation (8), that is, lines representing molecular weight species with different $f(M)$ values. Now looking at Fig. 6 we find that the points representing the first turbidity for the different species for each polymer concentration fall in practically a straight line (dotted in Fig. 6) and that these lines are parallel with each other. The angle coefficient for these lines is found to be -103.3. In Fig. 9 we can thus draw lines with this angle coefficient for different polymer concentrations (dotted), here calculated as a percentage of C_T^0, which in these experiments was kept at 0.5 mg per 100 ml. By using $(\Delta T)_i^\gamma$ as a parameter of the weight fraction that comes out of solution in the interval P^γ to ΔP, we can from this diagram find a representative $f(M)$ value for equation (17).

In this way all the factors in the right-hand part of equation (17) can be evaluated, and the weight fraction of each molecular species and its percentage part of the whole polymer can thus be calculated. For practical application a nomogram may be constructed where the numerical value of the denominator in equation (17) can be found for various alcohol concentrations and for different weight fractions of the molecular weight species in question (Fig. 10).

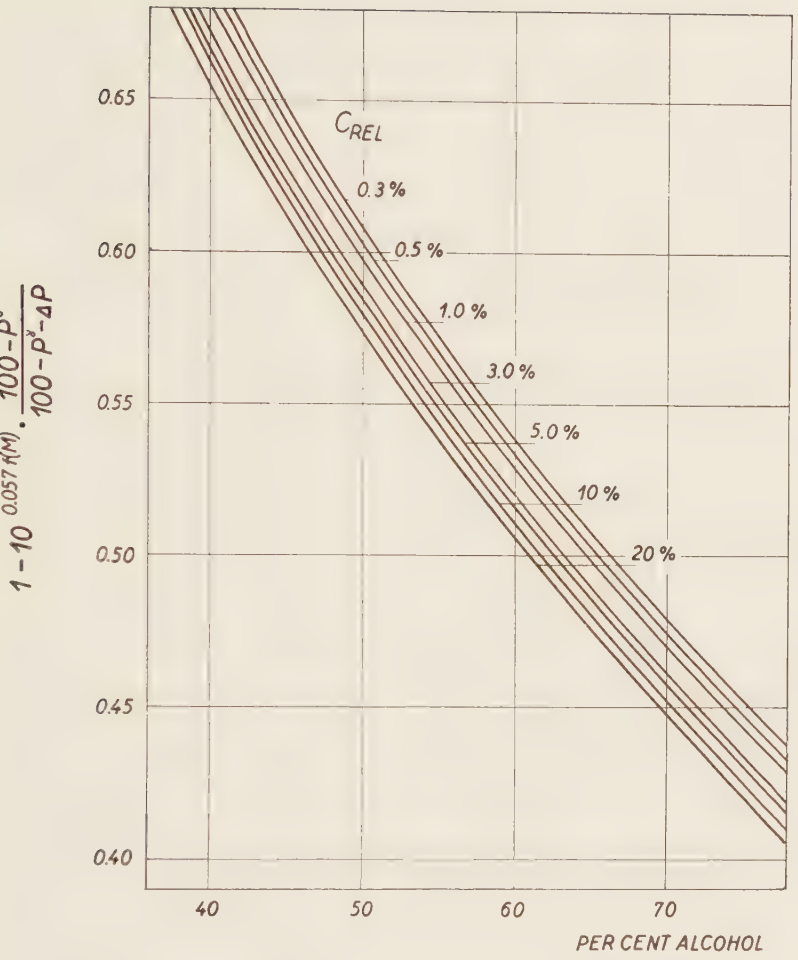

Fig. 10. Nomogram for the calculation of the denominator of equation (17) from the relative concentration of a molecular species, expressed as per cent of the total polymer, and the precipitant concentration.

The next step is to find the definitive $f(M)$ and the corresponding molecular weight for each fraction obtained. Solving for $f(M)$ in equation (15) we have:

$$f(M) = \frac{-P^{\gamma}}{0.057 \log \frac{100\,\frac{C_i^0}{C_T^0}\left(1-\frac{P^{\gamma}}{100}\right)C_T^0}{10^{\frac{1.114}{0.057}}}}. \qquad (18)$$

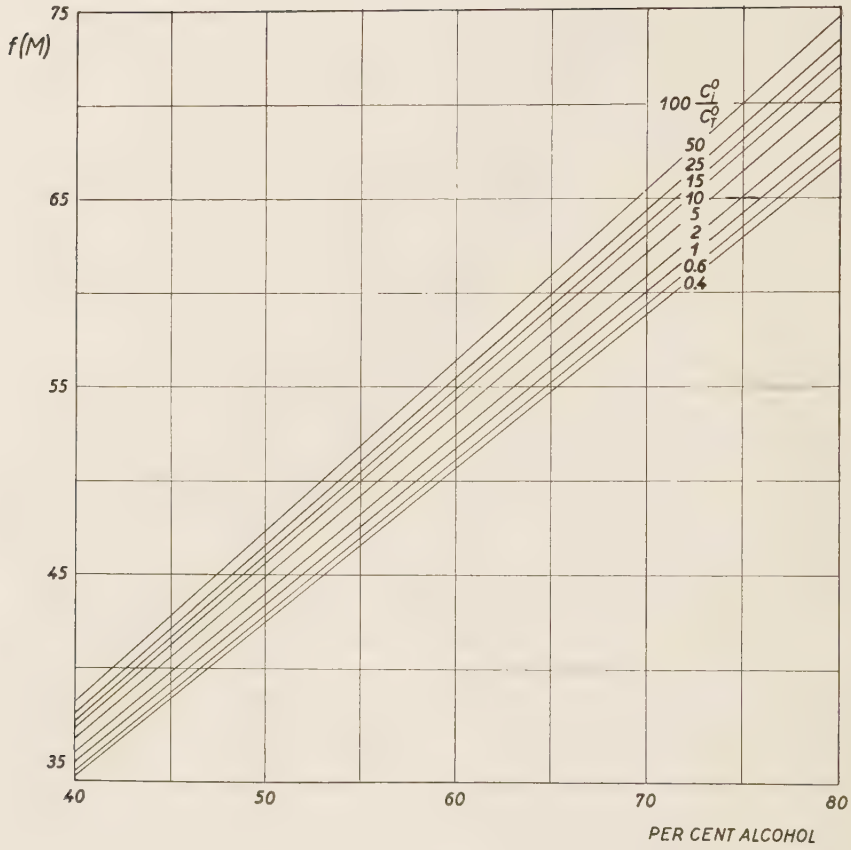

Fig. 11. Nomogram for the calculation of the $f(M)$-value of a molecular species from its 100 C_i^0/C_T^0-value and the alcohol concentration.

In actual practice a nomogram is constructed by placing different values of P and $100\frac{C_i^0}{C_T^0}$ in equation (18). The result is seen in Fig. 11.

The $f(M)$ value found in this manner may then be transformed to molecular weight, intrinsic viscosity or diffusion coefficient by the correlations given above.

By repeating the entire procedure for each P (ΔP as a rule kept at 1%) we are able to calculate the approximate concentration of each molecular weight species in the dextran preparation under investigation.

The next step is to verify that the approximation to use ΔT as a

Table 4. *Concentration and $f(M)$ values demonstrating that the turbidity increment ΔT approximately can replace the relative concentration of each molecular species during the calculation procedure.*

Per cent alcohol %	Turbidity increment in % of final turbidity ΔT %	Without F_Z-correction				With F_Z-correction				
		First approximation		Second approximation		$.1T$ after corr. = $.1Z$ %	First approximation		Second approximation	
		Conc. %	$f(M)$-value	Conc. %	$f(M)$-value		Conc. %	$f(M)$-value	Conc. %	$f(M)$-value
47	3.8	3.31	42.65	3.30	42.65	6.21	5.56	43.10	5.52	43.10
48	3.8	3.34	43.55	3.34	43.55	5.90	5.35	44.00	5.32	44.00
49	4.1	3.66	44.50	3.65	44.50	5.98	5.48	44.90	5.46	44.90
50	4.3	3.89	45.50	3.84	45.45	5.94	5.51	45.80	5.50	45.80
51	4.6	4.22	46.45	4.21	46.45	5.94	5.56	46.70	5.56	46.70
52	5.4	5.04	47.50	5.03	47.50	6.00	5.70	47.60	5.68	47.60
53	5.5	5.20	48.45	5.19	48.45	6.12	5.92	48.50	5.90	48.50
54	5.6	5.35	49.40	5.35	49.40	5.85	5.73	49.40	5.72	49.40
55	5.5	5.30	50.30	5.30	50.30	5.47	5.40	50.30	5.38	50.30
56	5.4	5.28	51.15	5.30	51.15	5.15	5.15	51.10	5.13	51.05
57	5.0	4.95	52.00	4.96	52.00	4.60	4.65	51.85	4.64	51.85
58	4.9	4.91	52.85	4.91	52.85	4.33	4.42	52.70	4.40	52.70
59	4.7	4.76	53.70	4.76	53.70	4.06	4.18	53.50	4.18	53.50
60	4.2	4.30	54.45	4.29	54.45	3.53	3.68	54.20	3.68	54.20
61	3.5	3.63	55.10	3.62	55.10	2.88	3.02	54.85	3.02	54.85
62	3.3	3.43	55.90	3.44	55.90	2.66	2.82	55.60	2.82	55.60
63	2.9	3.05	56.55	3.06	56.55	2.29	2.45	56.30	2.46	56.30
64	2.6	2.77	57.30	2.77	57.30	2.02	2.18	57.00	2.19	57.00
65	2.5	2.69	58.10	2.69	58.10	1.90	2.07	57.75	2.08	57.75
66	2.3	2.50	58.85	2.51	58.85	1.73	1.91	58.50	1.91	58.50
67	2.2	2.41	59.65	2.42	59.65	1.63	1.82	59.25	1.82	59.25
68	2.2	2.44	60.50	2.45	60.50	1.60	1.80	60.10	1.81	60.10
69	1.9	2.13	61.20	2.14	61.20	1.37	1.56	60.70	1.56	60.70
70	1.8	2.04	61.95	2.05	61.95	1.28	1.48	61.50	1.48	61.50
71	1.7	1.95	62.70	1.96	62.70	1.20	1.40	62.25	1.40	62.25
72	1.7	1.97	63.60	1.98	63.60	1.19	1.40	63.05	1.41	63.05
73	1.4	1.64	64.15	1.64	64.15	0.97	1.15	63.70	1.15	63.70
74	1.2	1.42	64.80	1.42	64.80	0.83	1.00	64.30	1.00	64.30
75	1.0	1.20	65.40	1.20	65.40	0.68	0.82	64.80	0.82	64.80
76	0.7	0.84	65.70	0.84	65.70	0.48	0.58	65.20	0.59	65.20
77	0.4	0.48	65.75	0.48	65.75	0.28	0.34	65.25	0.35	65.25

parameter of ΔZ has been permissible. If we take the numerical value of the concentration of species M_i that has been found by the approximation procedure above, and with a second approximation by means of the nomogram (Fig. 10) find a new value for the denominator of equation (17) and then recalculate the relative concentration of species M_i, the new result should more closely approach the true value. As this procedure is repeated each result will approach the true value asymptotically.

The left-hand part of the Table 4 contains the results of such a calculation for a dextran preparation (DRI 198). It appears that the results for the relative concentrations as well as for the $f(M)$ values are so close to each other that the approximation made above must be considered permissible.

Relation between turbidity and amount precipitated

An important question that now has to be discussed is the relationship between the turbidity increment at each alcohol addition and the weight of the dextran that thereby comes out of solution.

(a) By measuring the turbidity of a very fine dispersion of bentonite (an aluminium silicate preparation) at different degrees of dilution, it was found that the deflection of the galvanometer was a simple function of the concentration of the suspension. This shows the linearity of the system used.

(b) When analyzing by turbidimetric titration one and the same dextran preparation several times, the final turbidity obtained at the end of each titration will sometimes amount to different values. In preliminary experiments using a dextran concentration of 0.5–1.0% in the cuvette this variation of the final turbidity was sometimes rather wide. By successively lowering of the concentration of the dextran solution to be titrated and by increasing the sensitivity of the optical measuring system it was found that this variation could be practically eliminated. When using an initial dextran concentration of 0.0005%, this variation in the final turbidity could be kept within tolerable limits, as a rule less than $\pm 5\%$.

Furthermore, the above-mentioned variability in turbidity does not influence the final results in any measureable degree because of the following fact. When the absolute turbidity values read at the titration experiments are calculated as per cent of the final turbidity, it is found that even if there is a relatively large deviation in final turbidity in two experiments, these percentage values will yield practically identical curves.

(c) Another question is the constancy with time of the turbidity obtained at each addition of precipitant. A series of experiments analyzing this circumstance showed that immediately after the addition of alcohol there was a short equilibration period during which the mixing heat was eliminated through the cooling system and during which there was an

increase in the deflection of the galvanometer. After a lapse of 2 minutes the temperature in the cell had returned to normal level (20° C) and the galvanometer deflection had attained a constant value which in separate experiments remained constant for several hours. This constancy was controlled at different alcohol concentrations with the same result.

(d) To get an impression of the dimensional character of the dextran aggregates formed in the cuvette during a titration, some experiments were carried out. Material from the cell contents in different titration experiments was observed in a microscope under dark field illumination. The aggregates were seen as small brilliant spheres of uniform size. Their diameter was estimated to be 0.3 to 0.5 $\times$ 10^{-4} cm. No larger particles were observed. It could not be decided if smaller particles of dimensions less than the resolving power of the microscope existed. Thus the precipitated aggregates were shown to have dimensions of the same order of magnitude as that of the wavelength of the light from the mercury lamp. Accordingly, Rayleigh's law cannot be applied to this system, as this law is valid only for particles that are very small in comparison with the wavelength of the light.

(e) The quantitative relationships in the analytical system used may, finally, be tested by preparing a mixture of different narrow fractions in defined proportions, analyzing it as outlined above, and comparing the result with the theoretically calculated curve.

Thus, six "sharp" fractions of the type described on page 17, distributed at intervals of the molecular weight range 9,250 to 220,000, were mixed in pairs in equal proportions so that one of the fractions in the first mixture was identical with one fraction in the next mixture, the other component of which then was included in the following mixture, etc. In this way a chain of mixtures was obtained with each adjacent pair having one joint component. Each of the fractions and each of the fraction mixtures were then analyzed by turbidimetric titration and the results were transformed into distribution curves according to the principle outlined above. By comparing the curves of the individual fractions with those of the mixtures it was possible to calculate how much of the high-molecular fraction should have precipitated at a selected alcohol concentration on the plateau of the mixture curves. The results are shown in Table 5. However, it was found that the results deviated from the theoretically calculated figures, indicating a concentration of the high-molecular portion that is much lower than is really the case. This indicates that the assumption of Morey and Tamblyn (1945) that ΔZ equals ΔT does not apply in the dextran-water-ethanol system.

There are many reasons for this discrepancy between ΔZ and ΔT. Some of them will be discussed below (page 48). Here it suffices to point out this source of systematical error in the method. In order to eliminate this deviation it thus becomes necessary to introduce a correction procedure.

Table 5. *Control experiments with binary mixtures of dextran fractions.*

Exp. No.	Mixture of fractions, mol.w and $f(M)$-value		Limit at alcohol conc., %	High-molecular fraction in per cent of the total mixture		$\frac{F_Z^H}{F_Z^L}$	F_Z^L	F_Z^H	A, after corr. (C)
	Low	High		Experimentally found value (A)	Theoretically deduced value (B)				
1	9.250 64.0	85.700 45.0	60	25.5	41.5	2.06	1.50	3.09	40.5
2	9.250 64.0	138.000 42.3	59	26.0	43.5	2.37	1.50	3.56	44.5
3	14.500 58.9	138.000 42.3	55	23.7	40.7	2.20	1.62	3.56	40.7
4	14.500 58.9	220.000 39.1	56	21.6	42.5	2.67	1.62	4.32	42.0
5	34.700 51.4	220.000 39.1	50	22.2	38.0	2.14	2.02	4.32	37.5
6	9.250 64.0	220.000 39.1	60	42.3	45.0	2.86	1.50	4.32	45.0

Correction procedure

The correction factor to be applied must have different numerical values for different alcohol concentrations, showing a continuous decrease the higher the alcohol concentration becomes. By applying the following equation

$$A F_Z^H = \frac{B\,[A F_Z^H + (100 - A) F_Z^L]}{100}, \tag{19}$$

where A is the experimentally found value of the high-molecular fraction in per cent of the total mixture, B the corresponding theoretically calculated percentage, and F_Z^H and F_Z^L the correction factors for the high (H) and low (L) molecular part of the mixtures, inserting in this equation the data found in the experiments, we can solve the ratio F_Z^H/F_Z^L in each case.

If we now consider the most low-molecular fraction ($M = 9{,}250$, $f(M) = 64.0$) and arbitrarily choose a correction factor $F_Z^L = 1.5$ to be representative for this $f(M)$ value, we find in Table 5, experiment No. 1, that the correction factor ratio in this combination equals 2.06. F_Z^H for the other component in this mixture with $f(M) = 45.0$ as a representative value can now be calculated and is found to be 3.09. In experiment No. 2 we find the correction factor ratio to be 2.37, and by inserting the F_Z^L estimated in the first experiment, F_Z^H in the second mixture is found

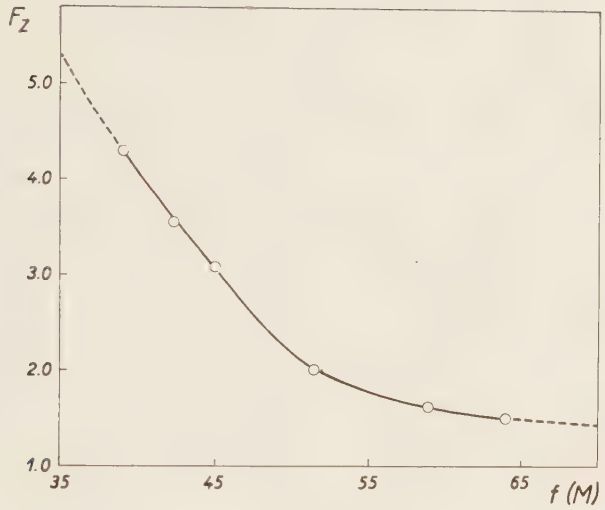

Fig. 12.

to be 3.56. Continuing in this manner we can calculate a series of correction factors for different representative $f(M)$ values (Fig. 12). Joining these points with a curve we obtain a nomogram from which the correction factor for any $f(M)$ can be found.

If we now apply this variable F_Z factor to each ΔT in the experiments with the binary mixtures described above we have the results shown in column C of Table 5. As can be seen, these results are in close agreement with the theoretically deduced figures shown in column B of the same table.

This function F_Z vs. $f(M)$ has now to be incorporated into the system used to transform the experimental data to a distribution curve. By using different numerical values of P and ΔZ in equation (17) or the nomogram (Fig. 10) we can find the corresponding $f(M)$ and from Fig. 11 the corresponding F_Z. We are thus able to make a nomogram for F_Z as a function of ΔT and P (Fig. 13). From this diagram we can find for each pair of ΔT and P in a titration experiment the F_Z that should be applied.

If this F_Z-correction procedure now is applied to the titration experiment with dextran preparation DRI 198 reported in Table 4, and the approximation procedure described above is again tested, it is found that the approximation still is permissible (Table 4).

Definitive procedure

We may now summarize the definitive procedure for calculating the experimentally found data of the turbidity titration experiments as follows:

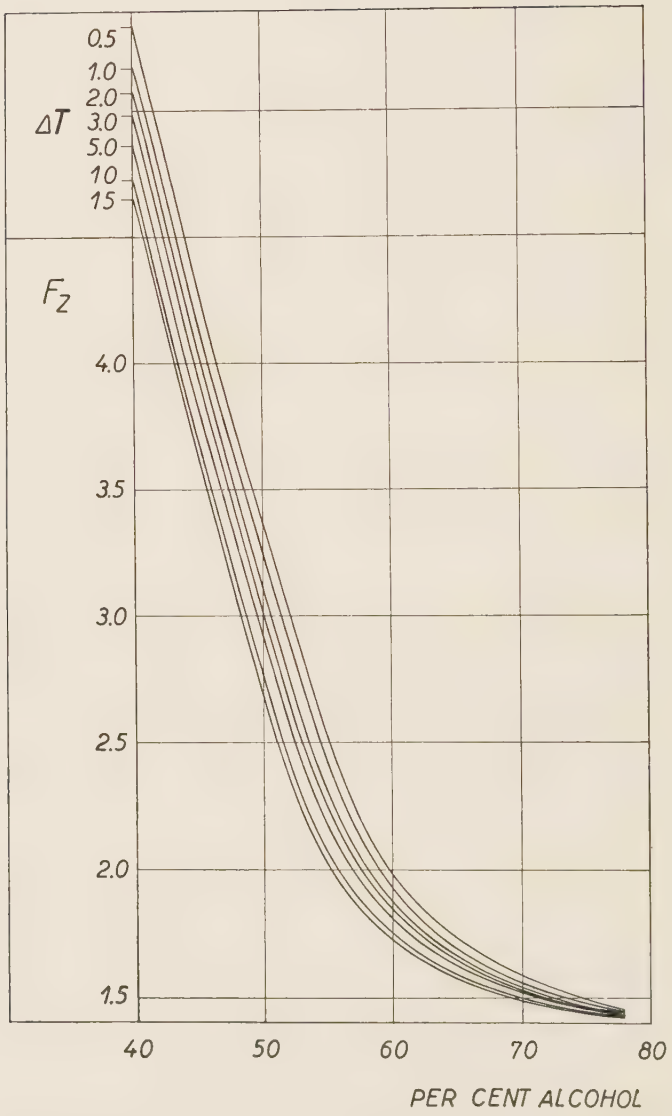

Fig. 13. Nomogram for the calculation of the correction factor Fz from ΔT and alcohol concentration.

(1) The relative turbidity at each titration step is corrected for the dilution caused by addition of precipitant and calculated as per cent of the final turbidity.

(2) These figures are plotted against the alcohol concentration at each reading to obtain a "titration curve".

(3) Starting from the point where the precipitation begins, that is, where the extrapolated titration curve cuts the abscissa, the turbidity increment ΔT corresponding to each alcohol increment ΔP (usually 1 %) is measured graphically.

(4) From Fig. 13 the corresponding F_Z is found by which ΔT has to be multiplied. Each $F_Z \Delta T$ is calculated as per cent of $\Sigma F_Z \Delta T$.

(5) Placing these values into the diagram (Fig. 10) we obtain the denominator of equation (17). The ΔZ-value obtained above is divided by this figure and then we have 100 C_i^0/C_T^0.

(6) In Fig. 11 the corresponding $f(M)$ value at the alcohol concentration in question is found, and by means of Fig. 7 this value may be transformed to molecular weight.

(7) The percentage of each part of Σ 100 C_i^0/C_T^0 is calculated and the molecular weight distribution curve can be plotted.

Control of the method

In order to test the reproducibility of the results that are obtained by the turbidity titration procedure, a series of experiments were performed on different days using the same dextran preparation (DRI 191). In addition two of the experiments (Nos. V and VI in Table 6 and 7) were done after treatment of the substance with picric acid as is done in the isolation method described on page 23.

The results are shown in Tables 6 and 7. In Table 6 the variation of the molecular weight values obtained are calculated assuming that the concentration values obtained were true. Table 7 shows the variations of the concentration values for the different molecular weight classes when the molecular weight estimation is assumed to be correct. The numerical differences are calculated as percentages of the means of the different groups and the standard deviation is given in per cent. The molecular weight values show a standard deviation of 0.5 to 5.2 % for the different molecular classes, and the concentration values show a standard deviation between 4.1 to 8.4 % of the means. When comparing with other methods for the same type of determinations, the reproducibility here achieved is considered to be fairly good.

As an illustration some demonstrative control experiments will now be reported and discussed. Fig. 14 shows the final distribution curves obtained in experiment No. 1 in the series with the binary mixtures, where two narrow dextran fractions with $f(M)$ values of 45.0 and 64.0 respectively were analyzed both separately and in a mixture of equal parts. The dotted lines show the distributions of the separate components, and the solid line that of the mixture. The actual per cent figures for the latter have been multiplied by 2 in order to make a more convenient

Table 6. *Molecular weight values* $M(\times 10^{-3})$ *and their differences* d *(in %) from the mean at fixed concentration values in six different experiments on the same dextran preparation.*

Conc. %	I		II		III		IV		V		VI		Mean		S.D.[1] %
	M	d	M	d	M	d	M	d	M	d	M	d	M	d	
10	9.8	+2.1	9.5	−1.0	9.7	+1.0	9.7	+1.0	9.7	+1.0	9.4	−2.1	9.6	1.4	1.6
20	12.7	+1.6	12.2	−2.4	12.5	0.0	12.8	+2.4	12.6	+0.8	12.1	−3.2	12.5	1.7	2.2
30	16.0	−0.6	15.3	−5.0	15.7	−2.5	16.7	+3.7	16.8	+4.4	16.0	−0.6	16.1	2.8	3.6
40	19.6	−3.0	18.8	−7.0	19.7	−2.5	20.8	+3.0	21.7	+7.4	20.6	+2.0	20.2	4.1	5.1
50	24.0	−3.8	23.2	−6.8	24.5	−1.6	25.7	+3.2	26.8	+7.6	25.5	+2.4	24.9	4.2	5.2
60	29.9	−2.9	29.0	−5.9	30.6	−0.7	31.7	+2.9	32.9	+6.5	30.8	0.0	30.8	3.2	4.3
70	38.9	−1.3	37.7	−4.3	39.8	+1.0	39.9	+1.3	41.0	+4.1	39.4	0.0	39.4	2.0	2.8
80	52.5	−0.4	51.3	−2.7	53.3	+1.1	52.3	−0.8	53.7	+1.9	53.0	+0.6	52.7	1.3	1.6
90	72.0	+0.3	71.7	−0.1	71.7	−0.1	71.3	−0.7	72.0	+0.3	72.2	+0.6	71.8	0.4	0.5
98	108.0	0.0	105.0	−2.8	107.5	−0.5	109.5	+1.4	112.0	+3.7	105.0	−2.8	108.0	1.9	2.5

[1] The standard deviation S.D. in Tables 5 and 6 is calculated according to the formula

$$\text{S.D.} = \pm\sqrt{\frac{\Sigma d^2}{N-1}},$$

where d equals the deviations from the mean, and N the number of observations.

Table 7. *Concentration values C of the different molecular weight groups and their differences d (in ‰) from the mean at fixed molecular weight intervals in six different experiments on the same dextran preparation.*

Mol.w. $\times 10^{-3}$	I		II		III		IV		V		VI		Mean		S.D.[1] %
	C	*d*	*C*	*d*	*C*	*d*	*C*	*d*	*C*	*d*	*C*	*d*	*C*	*d*	
7– 17	33.0	+1.5	35.0	+ 7.7	33.5	+3.1	30.7	−5.5	30.5	− 6.2	32.2	−0.9	32.5	4.2	5.3
17– 27	22.5	+5.6	22.0	+ 3.3	20.8	−2.4	21.8	+2.4	20.0	− 6.1	20.8	−2.4	21.3	3.7	4.4
27– 37	12.3	−7.3	12.4	−10.1	13.2	−4.4	14.5	+5.1	15.3	+10.9	14.6	+5.8	13.8	7.3	8.4
37– 47	8.1	−4.7	7.8	− 8.2	8.2	−3.5	9.1	+7.1	9.3	+ 9.4	8.4	−1.2	8.5	5.7	6.9
47– 57	6.2	−4.6	6.0	− 7.7	6.4	−1.5	7.0	+7.7	7.0	+ 7.7	6.3	−3.1	6.5	5.4	6.5
57– 67	5.1	−1.9	4.8	− 7.7	5.6	+7.7	5.2	0.0	5.5	+ 5.8	5.2	0.0	5.2	3.9	5.6
67– 77	4.1	0.0	3.9	− 4.9	4.3	+4.9	3.7	−9.8	4.3	+ 4.9	4.4	+7.3	4.1	5.3	6.7
77– 87	2.8	−0.3	3.0	+ 6.0	2.8	−0.3	2.7	−4.6	2.6	− 6.0	3.1	+9.5	2.8	4.5	6.1
87– 97	2.0	+4.2	2.0	+ 4.2	1.9	−1.0	1.8	−6.3	1.8	− 6.3	2.0	+9.4	1.9	5.2	6.4
97–107	1.3	+2.4	1.3	+ 2.4	1.3	+2.4	1.3	+2.4	1.2	− 5.5	1.2	−5.5	1.3	3.4	4.1
107–117	0.8	+6.7	0.7	− 6.7	0.7	−6.7	0.8	+6.7	0.8	+ 6.7	0.7	−6.7	0.8	6.7	7.3

[1] See note to Table 6.

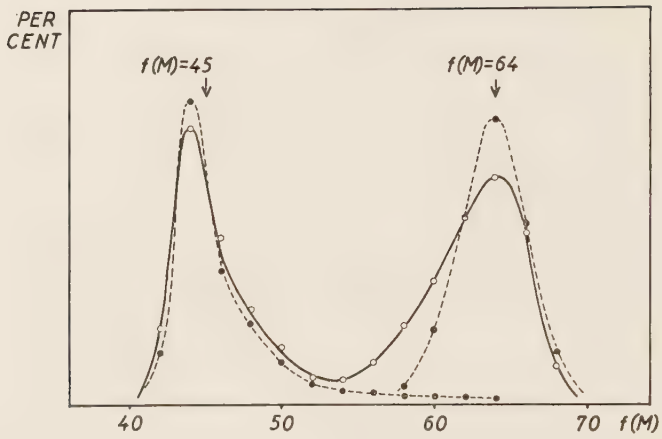

Fig. 14. Distribution curve for two dextran fractions analyzed separately and as a mixture of equal parts. The $f(M)$-value of the fractions are indicated by arrows.

comparison. The representative $f(M)$ values of the fractions in Fig. 14, shown by arrows, coincide rather well with the peaks of the curves, and the 1:1 composition of the mixture is clearly indicated.

In Fig. 15 another example is given. In this experiment a preparation of "clinical" dextran (Pharmacia DRI 201, $[\eta] = 0.255$) was analyzed according to the turbidity titration technique as described above, and the more conventional technique of fractional precipitation with subsequent determination of the intrinsic viscosity of the isolated subfractions.[1]

In using this latter technique, it is impossible because of practical considerations to carry out the fractionation at such a low dextran concentration as in the turbidity experiments. The fractional precipitations were performed at two different dextran concentrations: 6.0 and 0.6%, using ethanol as precipitant, 9 and 10 subfractions were obtained respectively. The intrinsic viscosity of each subfraction was determined following the same procedure as outlined on page 18. The final data are plotted in Fig. 15.

At first the deviation of the results of the three experiments seems rather large. The turbidity titration curve (0.0005%) indicates a more low molecular composition of the preparation than do the fractionation curves. This discrepancy may be explained in the following way.

In fractionation experiments using high concentrations of the polymer, coprecipitation phenomena as a rule appear. The less soluble, high-mo-

[1] These fractionation and viscosity measurement experiments were performed by Mr. A. Emneus, Pharmacia Ltd., Uppsala, who has very kindly made the results available to the author.

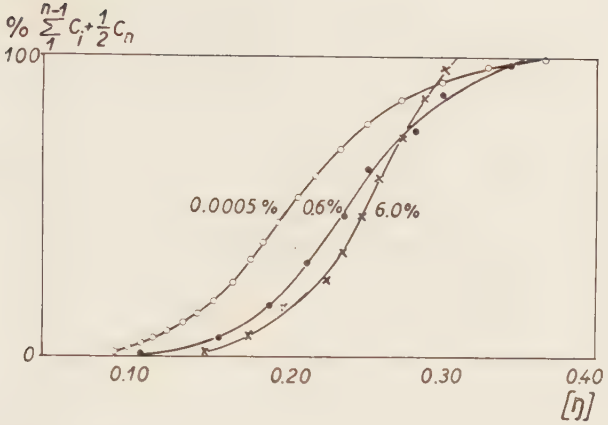

Fig. 15. Integral distribution curves for dextran DRI 201. The white dots indicate the results obtained by turbidimetric titration, the black dots and the crosses those obtained by conventional fractionation at 0.6 and 6.0% dextran concentration and subsequent viscosity measurement of the fractions.

lecular part of the substance will, when precipitating, "trap" a part of the still dissolved low-molecular species within the swollen, gel-like precipitate. Similarly at high polymer concentration part of the still dissolved molecules are mechanically entangled in the larger molecules and will thus follow them when they precipitate. Furthermore some consequences of the solubility limit law described above will contribute in the same direction (this is discussed below, see page 51). The effect of these various circumstances is that the more high-molecular fractions after isolation will show a contamination with the low-molecular part of the substance. From this it follows that the first fractions that come out of solution will show an intrinsic viscosity that is somewhat lower than would be the case if the fractions were more clear-cut and not contaminated. A further consequence of this is that the apparent concentration of the low-molecular fractions appears erroneously too low.

All of these coprecipitation phenomena show a tendency to decrease when the fractionation procedure is applied at lower polymer concentration. It is apparent that the lower the concentration, the more will the final curve approach the true distribution curve. With regard to this discussion the turbidity titration technique appears to be preferable to the conventional fractionation procedure, because it can be performed at a very low polymer concentration, and because no actual isolation of the fractions is needed as the precipitate is "weighed" optically while still in suspension.

However, even at very low concentration of the polymer a fractional precipitation will not yield an absolute separation of the different molec-

ular species. From the thermodynamic point of view there must always exist an equilibrium between the two phases in a precipitation system, and thus all species must always be present at least in small amounts in both phases and at all concentrations of the precipitant.

As no reference preparation of dextran exists, the true molecular weight distribution of which is exactly known, there is no direct way to test the accuracy of a method of this kind but to refer to the results of other methods, which themselves have relative large errors. The results that are obtained by turbidity titration can thus not be subjected to an exact test. From the experimental data it appears, however, that this method seems to picture the molecular weight distribution in a dextran sample with a fair degree of accuracy.

Discussion

The turbidimetric measurement.—The intensity of the light that is scattered by a turbid suspension in a direction perpendicular to that of the incident light beam is a function of several different factors, for example, the dimensions and number of the suspended particles, the difference of the refractive indices of the two phases, the wavelength of the light, etc.

In the turbidimetric titration procedure some other factors must also be considered. Through addition of alcohol there will be a dilution of the cell contents, which is to some degree counteracted by the contraction of the alcohol-water mixture. The refractive index will show a gradual change. The character of the precipitate will change with the concentration of precipitant: the higher this concentration the less hydrated and the more dense will be the precipitate. The aggregates formed at different alcohol concentrations will contain molecules of different molecular weights and cannot be presumed to be of uniform character. There may be a tendency of the aggregates to agglomerate as a function of time, that is, a kind of aging of the precipitate. Likewise, the presence of electrolytes will affect the results to a certain degree.

A theoretical analysis of the influence of all these factors on the turbidity obtained is a very complex problem (cf. Mie, 1908, Teorell, 1930, Ryde, 1931, and Casperson, 1932, 1933) and is not within the scope of this investigation. However, from the complexity of the system it appears that the assumption made by Morey and Tamblyn (1945), that the turbidity of the suspension after dilution correction is linearly related to the weight of the precipitate, can be valid only in exceptional cases. In any event, this approximation has not proved to be applicable to the dextran-water-alcohol system. However, by the application of a properly verified empirical correction procedure it has been possible to compensate for this source of error in the measurements.

The precipitation system.—By considering the relative energies in the

two phases of a precipitation system and applying a Bronsted-Boltzman expression for such energies, Schulz (1937) deduced the following equation:

$$C^{\gamma} = K \cdot e^{-(A+BP)(M/RT)} \tag{20}$$

where K, A and B are constants specific for the system used, M and T functions of molecular weight of the polymer and of temperature, respectively, and R the gas constant.[1] At constant temperature this expression leads to two consequences: (1) at constant degree of polymerization, the concentration of precipitant at saturation (P^{γ}) is linearly related to the logarithm of the polymer concentration (C^{γ}); (2) At constant polymer concentration, the concentration of precipitant at saturation should be linearly related to the reciprocal of the molecular weight.

This theoretical approach of Schulz includes some erroneous assumptions that have been pointed out by Morey and Tamblyn (1947), among others. Schulz assumes that the activities may be replaced by the actual concentrations, which is questionable at high concentrations of the polymer and when the molecules are aggregated. Similarly, the assumption that the concentration of polymer in the precipitated phase is constant, is erroneous to some extent, because the higher molecular weight aggregates are more swollen than the precipitates of the shorter chains (cf. Boyer, 1952). Fortunately the errors cancel each other to some extent and when the theoretical deductions are experimentally tested a good agreement is achieved in most systems.

Thus, when applying the two consequences of Schulz's expression referred to above to the dextran-water-ethanol system here investigated, the first relation is found to be true (cf. equation (8), page 31). However, if the data are plotted according to the second relation at constant $C = 1.0$, P^{γ} now being equal to $f(M)$, a curvilinear relationship between $f(M)$ and $1/M$ is found.

In some systems investigated by Schulz and Jirgensons (1940) a similar deviation from the theory was found, and by taking the form and structure of the polymer molecules into consideration, they showed that this linear relationship between the concentration of precipitant at saturation and the degree of polymerization at constant polymer concentration and temperature was only applicable in the case of strictly linear, rigid molecules. They found that an equation of the type

$$P = K\,1/M^{a} \tag{21}$$

was more generally applicable, where a is a parameter of the form and shape of the molecule. For ramified and coiled molecules a will assume a value less than 1.

[1] Derivations of similar expressions from the thermodynamic point of view has been made by Huggins (1942), Flory (1944) and Scott (1945).

An expression of this type agrees well in the case of dextran (equation (7), page 31), and a here assumes a value very close to $^1/_3$, thus indicating a compact and more or less globular shape of the dextran molecules.

A condition precedent for the applicability of Schulz's theoretical deductions is that the precipitate does not assume a crystalline character, which incident would affect the thermodynamic equilibrium between the two phases. When dealing with more or less branched, chain-like molecules such as dextran there is little chance that this circumstance will appear, and no signs of crystallinity of the precipitate have been observed in these experiments.

In the polymer systems investigated by Schulz and Jirgensons (1940) they found that the slope of the line, representing the relation between the concentration of precipitant and the logarithm of the polymer concentration, was inversely proportional to the degree of polymerization. In the system here investigated the same condition is found, that is, the factor K in equation (5) (page 29) is found to be a function of the size of the dextran molecules. When plotted against the solubility characteristic $f(M)$, a linear relationship is obtained.

This circumstance is, however, not considered by Morey and Tamblyn (1945) when they develop the turbidity titration method. Oth (1949), Harris and Miller (1951), Bischoff and Desreux (1951), and Mussa and Cernia (1951) have likewise neglected this circumstance. By doing so a systematical error is introduced, which can, however, be reduced by the introduction of a provisional $f(M)$ value as described on page 34.

Reverse order precipitation.—In a later paper Morey and Tamblyn (1946), however, discuss the problem treated in the preceding paragraph. They find that the appearance of this phenomenon is associated with the nature of the solvent-precipitant system used, and that in some systems this effect may be quite pronounced, resulting in a reverse order precipitation, which will be discussed below.

The presentation of a polymer-solvent-precipitant system as in Fig. 6 (page 30) is actually a very effective way to characterize such a system and to calculate the efficiency of fractionation that can be attained in each case. The greater the approaching tendency of the different fraction lines, the more dependent on initial polymer concentration will be the "resolving power" of the system. At the point of intersection of the fraction lines this power will show a minimum. At still higher polymer concentration a "reverse order precipitation" will occur, that is, the low-molecular part of the polymer will, paradoxically, show a tendency to come out of solution at lower concentration of the precipitant than does the high-molecular part. In this way the quotient between the angle coefficients of the fraction lines will constitute a parameter of the fractionation efficiency of the system as a function of the initial polymer concentration.

The inclination of the lines with respect to the abscissa will on the other hand demonstrate another character of a fractionation system. The less this inclination, the more of each molecular species will precipitate at each increment of the precipitant, and the greater this inclination the more precipitant has to be added to precipitate a molecular species as completely as possible. The degree of inclination of the fraction lines will thus constitute a parameter of the "precipitation power" of the precipitant in the solvent-solute system used.

In the dextran-water-ethanol system here investigated no phenomena of this type of reverse order precipitation have been observed, and from Fig. 6 it appears that the point of intersection between the fraction lines is situated at a polymer concentration that is too high to be attainable.

Influence of initial distribution of the preparation on fractionation efficiency.—The theoretical treatment in the preceding paragraph applies to the case when the different molecular species are made to be present in equal concentrations. Assuming that the polymer has a non-homogeneous distribution, which is generally the case, another kind of "reverse order" precipitation may occur, a phenomenon which is also a simple consequence of the saturation limit law and which can be easily predicted from equation (8) or Fig. 9 (page 33). We there find that the concentration of each molecular species as well as its molecular weight determined the precipitation point. Thus, a species of low molecular weight may begin to precipitate before one of high molecular weight, if the former initially is present in much greater amounts. From this it is evident that the starting distribution of the polymer plays a part in this respect and that abrupt changes or multiple peaks in the distribution curve may affect the results.

The non-fractionated dextran preparations used in these experiments showed, however, no abrupt changes of this kind, but when considering the "sharp" fractions used in the calibration procedure this phenomenon may presumably occur. In this instance this is actually a distinct advantage, because in this way the "sharp" fractions, in reality being far from homogeneous in composition, are "sharpened", so that the solubility limit attained as typical for a fraction, more closely corresponds to the representative part of it, that is, the part which determines its intrinsic viscosity, which in these experiments is used as a parameter of the molecular weight.

General comments.—In conclusion it may be said that by applying the theories of Schulz (1937) and Morey and Tamblyn (1945) it has proved possible to develop a method for the determination of the molecular weight distribution of partially degraded dextran with a comparably good reproducibility and yielding results with a fair degree of accuracy.

Criticism has often been directed against the accuracy and applicability of turbidimetric procedures in many respects, and much of this criti-

cism has been well founded. A common cause of the failure, in my opinion, is that in most methods which make use of turbidity measurements, the concentration of the substance in question has been kept too high, in that way obtaining a heavy precipitate of high density easily measurable by a common photometer. By selecting a sufficiently low concentration range, the system will gain in stability. In this way another advantage will also be attained: the procedure will consume only a small amount of the material to be tested, and will thus become a micro method.

CHAPTER 4

Experiments

Introduction

In the following chapter some physiological experiments on the renal elimination of dextran in humans and animals will be given. The chemical and physico-chemical methods used are, unless otherwise stated, those described in Chapter 3.

In order to give a more general picture of the dynamics of the dextran excretion process some human experiments using common clearance technique are reported first. An account will then be given of the maximal molecular weight of dextran that in normal conditions is able to pass through the kidney into the urine in some animal species. Similarly, data for normal humans and for some patients with kidney disorders and demonstrating proteinuria will be reported.

Furthermore, some experiments in dogs are reported, where the renal clearance of a low-molecular dextran fraction has been determined and compared with the simultaneously performed creatinine clearance; finally, the filtration rate for different molecular sizes of dextran relative to that of creatinine in normal dogs will be given.

The Urinary Excretion of Partially Degraded Dextran in Normal Humans

This series of experiments was made in order to obtain a general picture of the dextran excretion process in normal humans and its dependence on the time after the dextran injection.

It is known from the literature that during the first day after a dextran injection there is an excretion of dextran in the urine. Most of the dextran excreted is found in the urine from the first few hours after the injection. During the second day as a rule only minute amounts appear in the urine (Grönwall and Ingelman, 1945, Bull and co-workers, 1949).

In the series of experiments reported here this excretory process will be discussed in terms of renal clearance technique.

Table 8. *Clearance of dextran in normal humans at different times after injection.*

Subject, sex (m, f) weight, kg length, cm age, years corr. factor	Length of period min.	Diuresis, ml/min.	Urinary dextran excretion, mg/min.	Plasma dextran conc. mg%	Dextran clearance per 1.73 m^2 body surface	Time after inj., min.
B.K., m	21	1.9	31	98	32	43
67 kg	24	7.3	25	84	30	64
170 cm	22	11	15	68	22	88
37 yrs.	20	12	13	60	21	100
1.00	29	12	9.2	46	20	126
G.W., m	20	20	70	145	42	40
85 kg	20	14	28	109	23	60
182 cm	21	11	23	92	22	83
32 yrs.	22	13	17	81	19	102
0.89	21	11	10	68	13	123
	60	1.9	7.1	51	12	164
B.N., f	21	8.6	62	155	42	42
55 kg	21	12	30	130	24	60
103 cm	25	12	20	102	21	85
22 yrs.	24	11	14	90	17	108
1.06	21	11	12	78	16	132
B.R., f	21	8.3	59	152	39	46
60 kg	45	1.5	30	117	26	78
168 cm	21	2.2	16	91	18	112
36 yrs.	38	0.4	5.9	74	8	141
1.01						
K.W., m	62	1.5	37	169	20	60
70 kg	50	2.4	19	92	19	120
181 cm	180	8.2	14	76	17	141
23 yrs.	26	12	11	69	14	163
0.90						
E.J., f	20	4.6	63	128	49	32
76 kg	23	6.4	25	101	25	51
171 cm	20	7.2	21	78	27	72
39 yrs.	21	7.1	10	68	14	92
0.99	21	7.1	6.4	58	11	113
L.P., f	21	13	50	107	49	45
69 kg	22	15	29	90	32	62
171 cm	20	14	20	69	29	88
32 yrs.	21	12	14	65	21	108
0.99	22	11	9.4	55	17	129

Experiments

In these experiments dextran was administered as a single intravenous injection according to the principle of Alving and Miller (1940), and

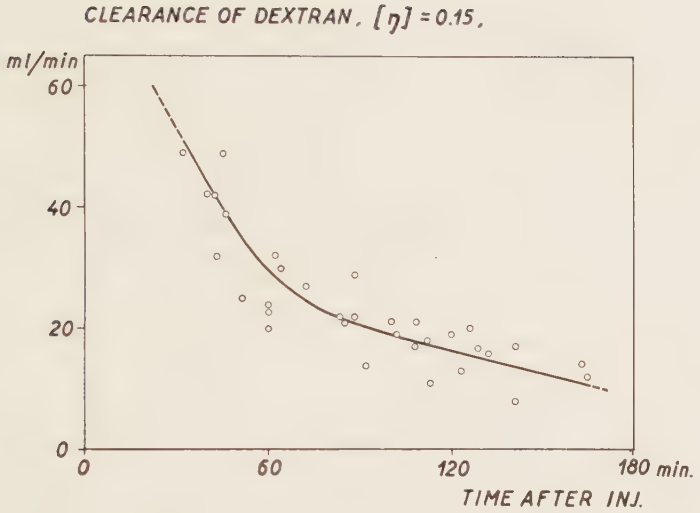

Fig. 16. Dextran clearance as a function of time after injection.

venous blood samples were used for analysis. No catheterization of the test subjects was made.

The dextran substance used consisted of a partially depolymerized dextran preparation (DR 694) that had an intrinsic viscosity at 20° C of 0.15, which corresponds to an average molecular weight of about 20,000. This relatively low-molecular dextran as compared to ordinary "clinical" dextran preparations, which usually have an intrinsic viscosity of about 0.25, was used in order to secure a rapid elimination for the purpose of the experiment. In preliminary tests it was found that about 75 % of this dextran preparation was able to pass into the urine of normal humans.

Seven normal fasting humans were given, between 8:00–8:30 a.m., 0.5–1 l of water perorally. 30 minutes later 10 g of the dextran preparation (100 ml of a 10% solution in normal saline) was slowly injected intravenously, the duration of the injection being about 10 minutes. After an equilibration period of about 30 minutes the urine was voided and the bladder was emptied as completely as possible. About 20 minutes later the bladder was emptied again and the urine was collected quantitatively. About three minutes before the middle of this urinary collection period (to allow for the delay of the urine when passing from kidney to bladder) a venous blood sample was drawn. Four to six similar clearance periods were followed in each subject.

The concentration of dextran in serum and urine was determined with

the anthrone method and the clearance was calculated according to the formula

$$C = \frac{U \cdot V}{P \cdot t} \qquad (22)$$

where C is the clearance of dextran (ml/min), U and P the dextran concentration in urine and plasma, respectively, in mg per 100 ml, V the volume of the urine in ml, and t the length of the urinary collection period (minutes).

No untoward reactions were observed in the test persons.

Results

The results are summarized in Table 8 and Fig. 16. The clearance values reported are reduced to 1.73 m^2 body surface area according to the nomogram of Peters and Van Slyke (1932).

It appears that there is a decrease in the clearance of this dextran preparation with time. The decrease is more rapid during the first hour after injection. During the first periods the clearance values are about 40–50 ml/min., that is about half the value of normal inulin clearance, and at the end of the experiment, i.e. two hours later, the clearance has decreased to 10–15 ml/min.

Discussion

The clearance values reported are rather scattered when plotted against the time that has passed after the injection. This is partly due to the biological variation in the different subjects, and partly due to experimental errors, which in these experiments were relatively large as no catheterization was used. However, when the observations are taken together they combine into a decreasing curved line.

Various mechanisms may be proposed to explain this slope of the clearance curve. Day (1952) has made some model experiments concerning the permeability of connective tissue. In some of his experiments, where such macromolecular substances as dextran and starch were used, he observed an increased resistance to flow through the membrane used (a mouse fascia). As an explanation of this retarding effect he suggests a blocking of the pores of the membrane through molecular impaction. However, it seems that in Day's experiments sufficient attention has not been paid to the oncotic pressure phenomena, which here would act in the same direction as would a clogging of the pores. Furthermore, the fact that dextran is a non-charged molecule decreases the possibility that a molecular impaction of the pores would be the predominant cause of the effect found.

In some clearance experiments in order to examine the influence of

dextran on renal function Fleming, Cargill and Bloom (1952) found no significant change of creatinine clearance after dextran injections when the plasma dextran concentration remained high. This result supports the assumption that molecular clogging of the pores according to Day would not be the cause of the decrease in dextran clearance reported above in this chapter.

Hoffman and Kozoll (1946) when studying the renal clearance of gelatin observed a similar progressive drop of the clearance values. They interpreted this as caused by the polydispersity of the injected material, the low-molecular fraction being rapidly cleared away in the beginning of the experiment, and thus its influence upon the observed clearance decreases with time. Beattie and Corcoran (1952) had similar experiences in experiments with a grass polysaccharide.

This explanation agrees well in the case of dextran. As will be shown in the last part of this chapter, the clearance of low-molecular dextran is of the same magnitude as the glomerular filtration rate, while the larger the molecules are, the less is their filtration rate. It seems therefore that the heterogeneity in molecular weight is here the main cause to the decrease in dextran clearance with time.[1]

The time course of this falling clearance curve will be discussed from a more theoretical point of view. In clearance experiments of the type reported here, three principal magnitudes of dextran molecules may be differentiated: (1) the most low-molecular part of the material, the molecules of which are able to pass the glomerular filter without any hindrance, (2) somewhat larger molecules, which are subjected to a relative restriction in their passage through the filter, and (3) the most high-molecular part, the molecules of which are unable to permeate the glomerular membrane for pure steric reasons. When using a chemical procedure such as the anthrone method for the analysis of urine and serum for dextran no informations as to the molecular sizes are, however, obtained. When the clearance value is calculated in this manner a kind of "average" clearance for the different molecular sizes results.

This "average" or total clearance is composed as a sum of the contributions from each molecular species. Suppose that at time t minutes after the injection we have a series of molecular sizes $M_1, M_2, \ldots M_n$ present in plasma, and let $P_1^t, P_2^t, \ldots P_n^t$ represent the plasma concentration of each species. Let $C_1, C_2, \ldots C_n$ denote the renal clearance of each species, and $U_1^t, U_2^t, \ldots U_n^t$ the amount that is excreted into the urine per time unit. Let C_T^t be the total, resulting clearance of all the different molecular species, that is, the clearance value we get when calculating with the total concentration of dextran in plasma and urine with no regard to the molecular weight distribution. Then we get

[1] When this article was in press it was observed that Raisz (1952) had made similar observations in dog experiments.

$$C_T^t = \frac{\sum_1^n (C_i \cdot P_i^t)}{\sum_1^n P_i^t} \tag{23}$$

The clearance C_i of the ith species is a function of the molecular magnitude and the character of the filter, and is independent of time, but P_i^t is dependent on the concentration of species M_i at $t = 0$, the elimination rate (= renal and extrarenal clearance), and the time that has passed after the injection, according to the equation

$$P_i^t = P_i^0 \cdot e^{-k_i t}, \tag{24}$$

where P_i^0 denotes the plasma concentration of M_i at injection, and k_i some function of the elimination rate. We thus get

$$C_T^t = \frac{\sum_1^n (C_i \cdot P_i^0 \cdot e^{-k_i t})}{\sum_1^n (P_i^0 \cdot e^{-k_i t})}. \tag{25}$$

From this expression it appears that the renal clearance of polymolecular dextran is a complex function of the separate clearance values for each molecular size, the molecular weight distribution curve of the substance at injection, the magnitude of the extrarenal elimination of each species, and the time that has passed after the injection.

A clearance experiment which uses a polymolecular test substance will thus give no immediate information concerning the state of permeability of the glomerular membrane. The analysis must be combined with an estimation of the distribution of the different molecular sizes in plasma and urine from the different clearance periods in order to give such information. In the last part of this chapter experiments of this character will be discussed.

Brewer (1951) reported a series of clearance experiments in rabbits in which three dextran preparations with different average molecular weight were used. In such experiments, however, the results obtained must necessarily be different at different times after injection and thus no definitive conclusions can be drawn.

Has this discussion any bearing on the behavior of other test substances used in clearance experiments? Some comments on this question with special regard to inulin will here be made.

A decrease in the clearance figures at falling plasma concentrations of the test substance is often observed. This concerns inulin as well as diodrast and para-amino hippurate clearance. This circumstance has

been the subject of much discussion and many theoretical explanations have been proposed. Brun, Hilden and Raaschou (1949) have studied this problem thoroughly, and they find that the error is due to significant differences in the concentration of the test substance in arterial and in systemic venous blood at rapidly changing plasma concentrations. If arterial plasma is used for analysis, this effect is mostly eliminated. As to inulin, this arterio-venous concentration difference was found to be relatively low, because the renal extraction of this substance is low compared to that of e.g. diodrast. Thus, the use of venous blood would only introduce a minor error as far as inulin clearance is concerned (cf. Laake, 1954).

Obviously, other factors may also influence the inulin clearance. From the discussion on dextran clearance given above, it is apparent that a possible polymolecularity of the inulin preparation used may affect the results in a similar way. The average molecular weight of inulin is reported to be different in different preparations, the figures varying between 4,500 and 7,300. While Ingelman (1946) has indicated that a preparation of inulin sulphate studied in the ultracentrifuge showed a polydispersity, no other information on this important question has been found in the literature.

In experiments that will be described in the following it will be shown that the largest dextran molecule which can pass without any restriction through the glomerular filter has a molecular weight of about 5,000 to 6,000. Allowing for differences in solvation and molecular shape, it is conceivable that in some inulin preparations the high-molecular part may have such dimensions that there will be a relative steric hindrance in the passage of these molecules through the glomerular membrane. This circumstance would cause an apparent inulin clearance value which would be lower than the glomerular filtration rate.

The Maximal Molecular Weight of Dextran Filtrable by the Kidney in Different Species and its Correlation to Proteinuria

In recent years there has been considerable interest in the study of experimental proteinuria and the closely related problem of tubular reabsorption and disposal of protein. The question of the participation of both glomerular and tubular factors in the pathogenesis of proteinuria is increasingly subject to experimental investigation.

In the series of experiments that is to be reported here some attempts have been made to establish, with the aid of a polymolecular dextran preparation, the upper limit for the passage of macromolecules into

the urine via the kidney in humans and some animal species, under normal conditions and in some kidney disorders demonstrating proteinuria.

Experiments

The dextran substance used in these experiments consisted of a "clinical" dextran preparation (Macrodex Pharmacia, DRI 200), which had an intrinsic viscosity at 20° C of 0.253. The test subjects were given an intravenous or intraperitoneal injection of a 6% solution of this substance in physiological saline. The urine that was excreted during 24 hours following the injection was collected and the urinary dextran was isolated according to the principle described in Chapter 3. The molecular weight of the most high-molecular part of the urinary dextran isolated was estimated by turbidity titration. As this fraction is present only in minute amounts the sensitivity and "resolving power" of the molecular weight determination method used will influence the results. The figures reported are therefore to be considered as relative values, and refer only to the highest molecular weight of dextran that is present in sufficient quantities to be detected by the technique here used.

The presence of protein in the urine samples was demonstrated qualitatively by the sulfosalicylic acid test. The amount of protein present was estimated in the first cases by Esbach's test, in which the protein is precipitated with picric acid and the volume of the sedimented precipitate is measured. Later on, a more exact quantitative method based on the biuret principle was used (Heyrovsky and Sochorova, 1951). Non-protein nitrogen in blood was determined according to the method of Rappaport (1949).

(a) *Animal experiments*

Guinea pigs.—Five guinea pigs were each given 2 ml of the dextran solution by injection, two animals intraperitoneally, and the rest intravenously. In three cases a light ether anaesthesia was given during the injection.

Rabbits.—Six white rabbits were each given an intravenous injection of 20 ml of the dextran solution. No anaesthesia was used.

Dogs.—One dog (St) was on two different occasions given an intravenous injection of 50 ml of dextran solution. Some weeks later it was found that this dog had developed proteinuria, presumably as a consequence of numerous bladder catheterizations with ascending pyelonephritis, and then a further dextran injection was given. Another dog (K) that likewise showed an occasional proteinuria, was also subjected to the same test.

Rats.—Seven white rats of the Wistar strain were given a 2 ml injection of dextran solution, one intraperitoneally and the rest intravenously. Four of the animals were given a light ether anaesthesia during the injection.

The urine excreted during the 24 hours following the injection was collected from all animals and analyzed as described above.

The animals showed no untoward reactions with the exception of the rats, which some minutes after the injection developed a condition with symptoms resembling allergic manifestations, such as swelling of the feet, nose and jowls with redness and itching of the skin, respiratory difficulties and stupor. The symptoms subsided slowly 2–3 hours after the injection. The reactions observed were similar to those described by Vorhees and co-workers (1951).

(b) *Human experiments*

Normal humans.—Ten normal human adults with no apparent symptoms of disease were in the morning given an intravenous infusion of 400 ml of the same dextran solution as was used in the animal experiments. The duration of the infusion was 20–30 minutes.

Patients.—Sixteen patients, suffering from different diseases in which the kidneys were more or less engaged and mostly demonstrating proteinuria, were subjected to the same test as were the normal humans.

No untoward reaction was observed in the human subjects. The urine excreted during the 24 hours following the infusion was collected from all the cases and analyzed as described above.

Results

The experimental results in the animal series appear in Table 9 and Fig. 17.

The largest dextran molecules detectable in urine collected during the 24 hours averaged in the normal animals: in guinea pigs 37,800, in rabbits 45,700 and in dogs 46,300. In the two dogs demonstrating proteinuria considerably higher molecular weights of dextran were found (80,000 and 62,000 respectively). All the rats had a slight proteinuria (a "normal" condition in the Wistar strain), and the largest urinary dextran molecule in this species averaged 62,700. The deviations of the individual values within each species were about 5–8% of the mean values given. The methodological error in the molecular weight determinations can be assumed to be about $\pm$ 5% (cf. page 44).

In the experiments with the normal human subjects the upper limit varied between molecular weights of 42,500–49,000, with 46,400 as an average value (Table 10 and Fig. 17).

The patients showed very scattered values in this respect (49,100–104,500). The figures for patients with proteinuria were consistently higher

Table 9. *The maximal molecular weight of dextran detectable in urine collected during 24 hours following the dextran injection in different animal species.*

Animal		Injection route[1]	Anaesthesia	Protein in urine	Mol.w. of largest dextran molecule detectable in 24 hours urine
Guinea pigs	1	i.p.	ether	−	36,200
	2	i.v.	ether	−	38,000
	3	i.p.	ether	−	35,000
	4	i.v.	—	−	39,500
	5	i.v.	—	−	40,500
				Mean	37,800
Rabbits	1	i.v.	—	−	46,000
	2	i.v.	—	−	45,700
	3	i.v.	—	−	48,200
	4	i.v.	—	−	45,700
	5	i.v.	—	−	44,000
	6	i.v.	—	−	44,700
				Mean	45,700
Rats	1	i.v.	—	+	61,700
	2	i.v.	ether	+	64,000
	3	i.v.	—	+	62,500
	4	i.v.	—	+	62,000
	5	i.p.	ether	+	62,200
	6	i.v.	—	+	66,000
	7	i.v.	ether	+	60,500
				Mean	62,700
Dogs	St. 1	i.v.	—	−	44,700
	St. 2	i.v.	—	−	48,000
	St. 3	i.v.	—	+ +	80,000
	K. 4	i.v.	—	+	62,000

[1] i.v. = intravenously, i.p. = intraperitoneally.

than those for the non-proteinuric cases. The largest dextran molecules were found in those cases which had abundant protein in their urine. No correlation was found to the non protein nitrogen content of the blood.

Discussion

A protein admixture of the urine can be brought about in several different ways: (1) an increased passage of protein over the glomerular membrane; (2) an overload of or an interference in the reabsorption of

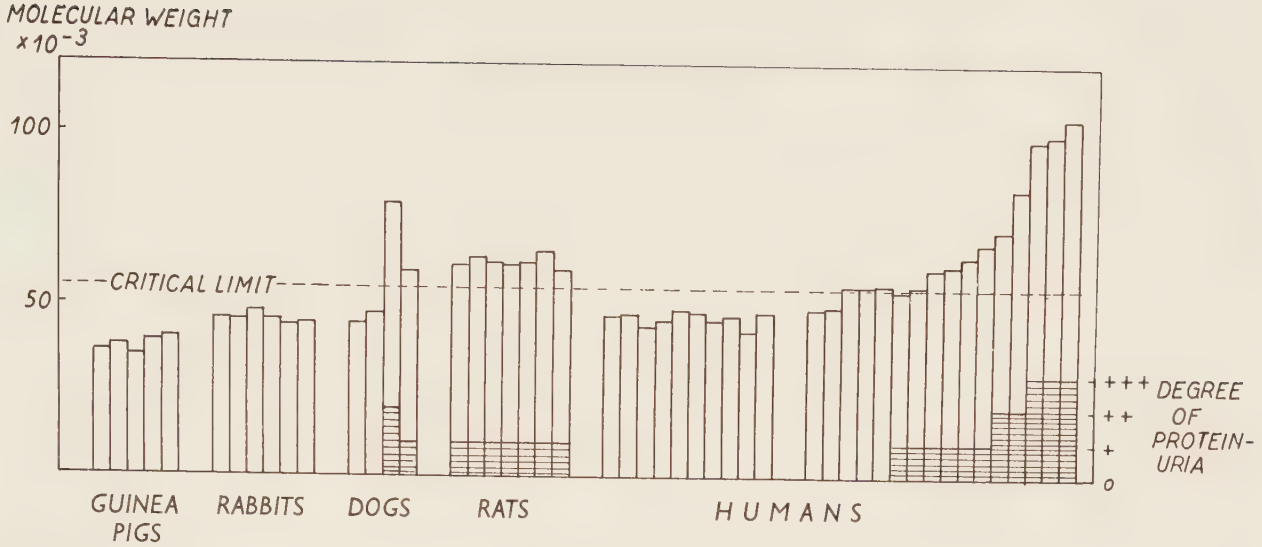

Fig. 17. The largest molecular weight of dextran detectable in urine collected during 24 hours after a dextran injection in some animal species and humans. The degree of proteinuria is indicated by the solid areas. At a molecular weight of about 55,000 there is a critical limit, above which protein appears in the urine.

Table 10. *Maximal molecular weight of dextran detectable in urine, collected during 24 hours after a dextran injection in humans.*

No.	Sex	Age, yrs.	Protein in urine		N.P.N mg %	Dextran, Mol.wt.	Diagnosis
			Qualitatively	Quantitatively %			
1	f	38	—	—		47,000	Normal humans
2	f	32	—	—		47,300	
3	f	33	—	—		43,600	
4	f	25	—	—		45,700	
5	f	24	—	—		49,000	
6	f	39	—	—		48,000	
7	m	28	—	—		45,600	
8	m	32	—	—		47,000	
9	m	35	—	—		42,500	
10	m	47	—	—		48,200	
					Mean	46,400	
M199/54	m	50	—	—	27	49,100	Bronchopneumonia
M2640/53	m	54	—	—	30	49,500	Arteriosclerosis
M259/54	m	70	+	0.11	45	54,200	Hypertension + org. cardiac failure
P3201/53	f	40	—	—		55,300	Burn, third degree
M2582/53	f	58	+	0.10	33	55,700	Chron.glomerulonephritis
P4211/53	m	21	—	—		56,800	Burn, second degree
P76/54	m	45	—	—		57,500	,, ,, ,,
M2528/53	f	53	+	0.10	88	61,200	Nephrosclerosis + nephrolithiasis
M432/54	m	69	+	0.05	32	62,000	Cancer prostatae
M2548/53	m	46	+	0.10	27	64,200	Acute glomerulonephritis
M2542/53	m	34	+	0.20	24	68,200	,, ,, (?)
K89/54	m	23	+ +	0.70	27	72,000	Amyloidosis + nephropathy
M135/54	m	26	+ +	0.80	48	84.300	Chron.glomerulonephritis
M2766/53	m	53	+ + +	0.90	38	98,500	Lymphogranulomatosis + nephropathy
M2547/53	f	65	+ + +	0.90	23	100,000	Amyloidosis + nephropathy
M2581/53	m	46	+ + +	1.20	39	104,500	Chron.glomerulonephritis

protein by the tubular epithelium; (3) an active excretion of protein by the tubular cells; (4) a passive transudation of protein through the urinary tract epithelium.

No evidence has ever been adduced that demonstrates an active excretion of protein by the tubular epithelium. A contamination of the urine by transudation through the mucous membranes of the renal pelvis, ureter or bladder, is more to be regarded as a source of error

and not a "true" proteinuria. Thus only the first two alternatives mentioned remain to be discussed.

An increased passage of protein through the glomerular membrane can be effected in two different ways, either by an increase in the permeability of the glomerular capillary wall or by the appearance in the blood stream of abnormal proteins of such molecular dimensions, that they are able to penetrate the normal glomerular membrane in sufficient quantities.

These different mechanisms seem to be sufficient to give a theoretical explanation to all observed conditions at which proteinuria occurs, whether originating from different diseases or experimentally induced. Some examples will here be given. In multiple myeloma an abnormal protein (Bence Jones protein) often appears in the blood stream. This protein has a molecular weight of about 38,000 (Svedberg and Pedersen, 1940), that is about half the value for serum albumin, and thus presumably being able to escape into the urine through a normal glomerular filter. At low concentrations of this protein in the blood most of the amounts that pass into the capsular space may be reabsorbed by the tubules. At higher blood concentrations of this protein more and more will pass into the glomerular urine until the reabsorptive capacity of the tubules is overloaded, and the protein will now appear in the definitive urine. Through the interference in the tubular function of this low molecular and thus more diffusible protein there will be a hindrance for the "normal" reabsorption of the slight traces of serum protein in the tubular lumen. In this way the definitive urine may contain, besides the abnormal Bence Jones protein, great amounts of the large molecular serum proteins, in spite of the fact that the permeability of the glomerular membrane is quite normal.

Experimental evidence supports such a theory as outlined above. Thus Ascoli (1902) showed that after injection of egg protein both this protein and plasma protein was excreted via the kidney. Gilligan, Altschule and Katersky (1941) made similar observations in experimental hemoglobinuria.

Many observations agree with the theory that the glomerular membrane normally allows small amounts of the plasma proteins to "leak" into the capsular space. Mörner (1895) found that the urine of normal humans contained 100–150 mg of protein per liter, an observation later confirmed by several authors. By raising the plasma protein concentration by homologous plasma injections, Claude Bernard (1859) observed that proteinuria could be induced in dogs. The observations of Ekehorn (1931) in his glomerular puncture experiments and recent observations by Terry and co-workers (1948) in dogs and Addis (1949) in rats have further corroborated the view that there exists a renal "threshold" for the normal serum proteins, so that when the plasma protein concentration is raised over a certain limit the tubular reabsorptive capacity

for protein will be overloaded and protein will appear in the urine. This seems to be a perfectly reversible condition, and no signs of renal damage have been demonstrated. Rather (1052) has recently given an excellent review of these and allied questions.

However, the most common cause of proteinuria is said to be the damage of the glomerular membrane, resulting in an increased permeability. While no direct proof that this is the case has been given, many observations indirectly support this theory. The analysis of the albumin/globulin quotient in serum and in urine in proteinuric cases (see e.g. Bing, 1030) does not rule out the possibility of different reabsorption of the various protein components during the tubular passage.

Summarizing the results of the dextran excretion experiments reported in this chapter the following has been found. In normal conditions there exists an upper limit for the penetration of macromolecules through the glomerular capillary wall. This limit varies slightly in different animal species. Over this limit the restriction to the passage of these molecules is great enough to prevent their passage in sufficient quantities to be detected by the methods employed. However, in those cases investigated where protein does pass into the urine, this upper limit is raised and the critical limit corresponds to a dextran molecular weight of about 55,000. It has furthermore been observed, that the more protein there is in the urine, the larger, as a rule, will be the molecules that can be found in the urine.

The explanation to this behaviour of the larger dextran molecules in the proteinuric cases can be either an increased permeability of the glomerular membrane, allowing larger dextran molecules to pass, or a tubular reabsorption of these larger dextran molecules that varies inversely to the degree of the proteinuria. However, in the following chapter it will be shown in clearance experiments that the tubular reabsorption of dextran, although demonstrable by histological technique, is rather insignificant and does not noticeably affect the clearance values found. This observation makes tubular interference in the problem here discussed unlikely but it cannot completely rule out such a circumstance. If we suppose a tubular interaction, it might be quite possible that the tubules, which normally would have reabsorbed sufficient quantities of the large dextran molecules to make them non-detectable by the method employed, in the proteinuric cases, where they are supposed to be "overloaded" by protein, leave these larger dextran molecules more or less unaffected during their passage through the tubules.

However, the presence in the tubular lumen of large quantities of low molecular dextran which owing to its higher diffusivity can be supposed to enter the tubular cells more easily, reduces the possibility that the observations here made can be explained solely by tubular activity. The glomerular factor seems therefore to be the predominant one.

About the rat series the following comments will be made. This species when injected with dextran develop a specific type of reaction (Vorhees and co-workers, 1951, Morrison and co-workers, 1951). Shortly after the dextran administration they show symptoms resembling allergic manifestations, such as edema of the nose and feet, respiratory difficulties and cyanosis, accompanied by hemoconcentration. These symptoms slowly disappear 2 3 hours after the injection. No reactions of this type have been observed in other animal species.

Edlund, Löfgren and Väli (1952) have studied this specific action of dextran, and they found that dextran when injected intradermally, caused an increased capillary permeability. Highly branched dextran molecules showed a greater effect than those with relatively unbranched chains. The administration of antihistaminics, local anaesthetics and alloxan, and pretreatment with cortisone showed an inhibitory effect.

From the experiments reported by the present author it cannot be decided, whether or not dextran has a similar action on the glomerular membrane. It can only be concluded that the kidney of this species, which normally permits protein to pass into the urine, allows larger dextran molecules to pass than is the case in the other species investigated.

In conclusion it can be said that the results of the experiments reported here support the assumption that proteinuria usually is the result of an increased permeability of the glomerular membrane. Furthermore, experiments of this type seem to be well suited for the analysis of other types of proteinuria of doubtful origin, e.g. orthostatic proteinuria.

The Renal Clearance of Low-Molecular Dextran in the Dog

All evidence favours the theory that dextran is excreted via the kidneys principally by filtration through the glomerular capillary wall. However, some histological investigations indicate that after a dextran injection granular matter of a dextran character is to be found within the tubular cells. Thus Friberg, Graf and Åberg (1951) and Persson (1952, 1954) demonstrated in rabbits that a transient deposition of dextran granules was noted in the cells of the convoluted tubules. In addition abundant dextran material was observed in the glomerular capillary loops, the subcapsular space and within the lumen of the tubular apparatus. From this picture no conclusion could be drawn whether the material within the tubular cells was the result of a tubular secretion or a tubular reabsorption of dextran.

To get an impression of the magnitude of this tubular interference in the dextran excretion process some experiments in dogs will here be re-

ported elucidating the renal clearance of a low-molecular dextran preparation and its relation to the glomerular filtration rate.

Experiments

The principle in these experiments was to determine the renal clearance of a very low-molecular dextran fraction and to compare the results with the simultaneously determined exogenous creatinine clearance.

In order to avoid any "molecular sieving" interference, the molecular dimensions of the dextran preparation used must be of similar magnitude as those of inulin. The molecular weight of inulin varies somewhat with the different preparations of the substance (mol. wt. 4,000–6,000; see e.g. Westfall and Landis, 1936).

The dextran substance (FDR 166) used in these experiments had an intrinsic viscosity at 20° C of 0.048, which corresponds to a molecular weight of about 4,000. The quantitative dextran determinations in serum and urine were made according to the method described in Chapter 3. Creatinine analysis of these fluids was performed according to the alcaline picrate method of Jaffé, as modified by Folin and Wu (1919). The non-creatinine chromogen error was depressed by the parenteral administration of large creatinine doses.

Five experiments in three different dogs, comprising a total of 15 clearance periods were performed. In two experiments the animals were anaesthetized, one with ether and one with pentobarbital. In three experiments in one dog no anaesthesia had to be used. Before the experiments the animals were hydratized perorally by stomach tube or by a continuous infusion of normal saline. Single injection technique was used with an intravenous injection if 0.2–0.6 g of dextran per kg of body weight and a subcutaneous injection of 0.05–0.3 g creatinine ("Pfanstiehl") per kg of body weight.

The first urine collection was begun 30–40 minutes after the dextran injection, each period lasting from 30–70 minutes. To allow for the delay of the urine to reach the bladder the venous blood samples were drawn 1–3 minutes before the middle of the urine collection periods. At the end of each period the bladder was emptied through an indwelling catheter and finally washed with a measured amount of physiological saline.

Results

The results of these experiments are shown in Table 11 and Figs. 18 and 19. From the table it appears that the clearance of this dextran preparation is close to that of creatinine. The quotient dextran/creatinine clearance is found to be 0.99 with a standard deviation of $\pm$ 0.06. Fig. 18 shows the dextran clearance as a function of creatinine clearance. The line has been fitted to the experimental results according to the method of least squares, and its equation was found to be:

Table 11. *The renal clearance of low-molecular dextran (average molecular weight 4,000) and exogenous creatinine in normal dogs.*

Dog Body weight kg	Length of period min.	Diuresis, ml/min.	Concentration in serum		Concentration in urine		Clearance		$\frac{C_{dx}}{C_{cr}}$	Dextran excretion per ml plasma cleared from creatinine mg/min.
			Dextran mg %	Creatinine mg %	Dextran mg %	Creatinine mg %	Dextran C_{dx} ml/min.	Creatinine C_{cr} ml/min.		
P	30	2.57	46.6	5.93	1930	266	106	115	0.92	0.49
27.3 kg	30	1.87	26.3	4.95	1580	296	112	112	1.00	0.29
	30	1.57	17.2	4.11	1020	240	93.1	91.6	1.02	0.16
	30	1.80	11.5	3.45	598	165	93.6	86.1	1.09	0.11
St	32	1.22	97.5	6.70	3490	262	43.7	47.7	0.92	1.07
8.1 kg	40	0.90	45.0	5.48	1765	232	35.3	38.1	0.93	0.40
	44	0.32	17.6	4.19	2020	484	36.7	37.0	0.99	0.16
,,	35	0.71	110.0	6.48	5490	341	35.4	37.4	0.95	0.99
	33	1.64	45.0	4.83	1075	115	39.2	39.0	1.01	0.44
	37	1.22	23.3	4.23	685	124	35.9	35.8	1.00	0.21
,,	50	1.10	65.3	5.51	2530	230	42.6	45.9	0.93	0.70
	74	0.60	17.6	3.98	1195	240	40.7	36.2	1.12	0.18
	30	0.38	5.7	3.17	656	334	43.7	40.0	1.09	0.06
A	30	0.37	58.3	3.34	10400	576	66.0	63.8	1.03	0.55
21.8 kg	30	0.37	32.6	2.88	6670	597	75.7	76.7	0.99	0.35

Average creatinine clearance: Dog P, 101.3; dog St, 39.6; dog A, 70.4.
Average dextran creatinine clearance ratio = 0.99 ± 0.06.

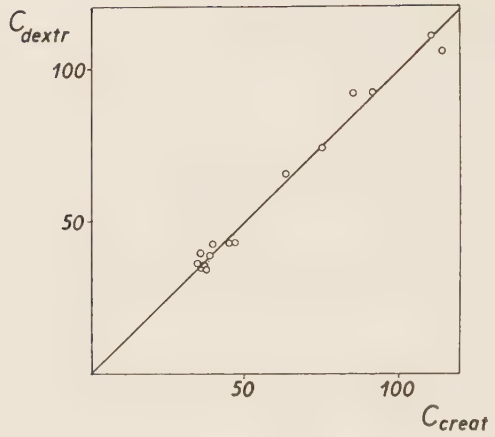

Fig. 18. Clearance of a low-molecular dextran preparation (mol. wt. 4,000) in normal dogs and its relation to the simultaneously performed exogenous creatinine clearance.

$$C_{dx} = 0.99\,C_{cr} + 0.72 \tag{26}$$

where C_{dx} and C_{cr} designate the clearance values for low-molecular dextran and creatinine respectively.

Fig. 19 shows the magnitude of the urinary excretion of dextran at different plasma concentrations. The excretion values are given in mg per minute per creatinine clearance unit, as the creatinine clearance is a much better measure of the "magnitude" of a dog from this point of view than is the body surface area. The regression line calculated according to the method of least squares was found to be

$$U = 0.0099\,P + 0.003 \tag{27}$$

where U equals the urinary dextran excretion reduced as described above and P the plasma concentration of dextran.

In some of the experiments a tendency to a similar decrease of the clearance values as that discussed on page 56 was observed, indicating the polydispersity of the dextran preparation. The anaesthesia applied in some of the experiments had no significant effect on the results (cf. Glauser and Selkurt, 1952).

Discussion

Dogs were selected as test animals in these experiments because in this species the exogenous creatinine clearance equals the inulin clearance and thus according to Smith (1951) constitutes a measure of the glomerular filtration rate. When creatinine is not administered the non-creatin-

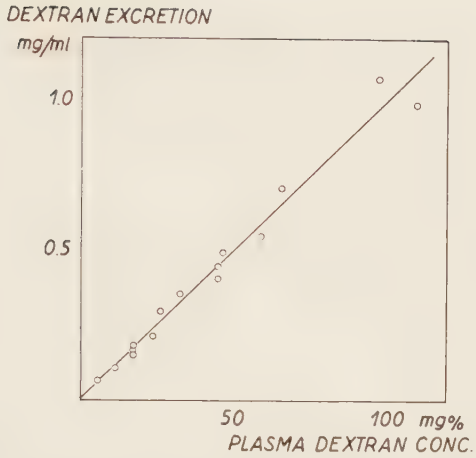

Fig. 19. Urinary excretion of low-molecular dextran, calculated as mg excreted per ml plasma cleared of its content of creatinine, and its relation to the plasma dextran concentration in normal dogs.

ine chromogenic substances in blood giving positive Jaffé reaction interferes, and erroneously low clearance values are obtained (Shannon, Jolliffe and Smith, 1932). The concentration of this non-creatinine interfering substance is generally less than 0.5 mg per 100 ml. By raising the blood level through the administration of large doses of creatinine, this source of error can be minimized.

The results of these experiments show that the clearance of the low-molecular fraction used is very similar to the creatinine clearance in the dog. The deviations from unity obtained are randomly scattered. Furthermore, the dextran excretion is found to be directly proportional to the plasma dextran concentration. If any tubular secretion or reabsorption had occurred, the regression line in Fig. 19 would have cut the ordinate at positive or negative y-values. The intercept of $+0.003$ found in the experiments is considered to be insignificant.

However, Kennedy, Hilton and Berliner (1952) recently suggested that a small part of the plasma creatinine is combined with the plasma proteins and is not freely diffusible. They found in dog experiments an average inulin/creatinine clearance ratio of 1.065.

If we apply the theory of Kennedy and co-workers in our case the result would be that a small part of the dextran is withheld by the kidney, either by restriction to passage through the glomerular filter or through reabsorption by the tubular cells. This latter circumstance agrees well with the histological findings referred to above.

When considering the great quantity of dextran that passes through the tubular lumen during the excretory process and which may amount

to figures of 5–10 per cent or higher, it is quite conceivable that a part of this dextran may enter the tubular cells by simple diffusion. This part must however correspond to a very low percentage of the bulk of dextran that passes within the tubular lumen. An active tubular reabsorption can be ruled out by considering the proportionality between dextran excretion and plasma concentration over the whole range investigated.

Thus it is found that low-molecular dextran, when passing through the tubuli to a low percentage by passive diffusion enters the tubular cells. This tubular interference seems to be relatively insignificant, as it is hardly measurable by common clearance technique.

It is rather difficult to get a similar quantitative estimation of the behavior of larger dextran molecules, those which are more or less restricted in their passage through the glomerular membrane. However, the histological picture of the tubular cells after injection of more high-molecular dextran fractions (Persson, 1954) does not support the conception that the tubular interference would increase with respect to these large molecules. In addition, since the low-molecular dextran preparation used in the experiments reported above shows only a very slight degree of migration into the tubular cells in spite of having a large diffusivity, it is not conceivable that somewhat larger and thus less diffusible molecules of the same polymer would enter the tubular cells more easily.

In conclusion, the experimental results here obtained indicate that the clearance of low-molecular dextran with an average molecular weight of about 4,000 is very close to the glomerular filtration rate, and that the histologically demonstrable accumulation of dextran granules in the tubular cells is relatively insignificant and causes no measureable deviation in the clearance experiments.

The Relative Filtration Rate of Different Molecular Sizes of Dextran in the Dog

It is evident from the preceding experiments that a dextran clearance study must comprise not only the quantitative estimation of this substance in serum and urine, but also a determination of the relative concentration of all the participating molecular species of the substance in both of these fluids. In this way complete information on the dynamics of the dextran excretion process may be obtained. This also makes possible a definite statement concerning the renal excretion of a polydisperse substance and indirectly reveals information about the renal filtration process and the action and structure of the glomerular membrane.

In this section some experiments of this character will be described, and the results will be discussed in terms of molecular sieving and similar concepts.

Experiments

The test substance used was a dextran fraction (FDR 146) of the type described in Chapter 3, with a distribution of molecular sizes so selected that the most low-molecular part would pass through the glomerular membrane relatively freely, while the most high-molecular part was in general retained by this filter. The preparation had an intrinsic viscosity of 0.14 at 20° C, which corresponds to an average molecular weight of about 19,000.

Fasting mongrel dogs, trained to lie quietly on the table when being catheterized, were hydrated by stomach tube or by intravenous infusion of normal saline. In some experiments anaesthesia, in the form of pentobarbital sodium given intravenously, was used. Urine was collected by an indwelling catheter. Each urine collection period was terminated by washing the bladder with normal saline and by injection and re-expression of air. An indwelling needle was used to remove blood from one of the paw veins. To compensate for the delay time, i.e. the time for the urine to reach the bladder from the glomerulus, the blood specimens were taken a few minutes before the beginning and the end of the urine collection period. This interval was checked shortly after the urine collection period by injecting a small amount of fluorescein sodium intravenously and recording the time at which fluorescence appeared in the urine. This was observed by inserting a glass syringe in the bladder catheter and by forcing normal saline back and forth, thereby securing a rapid mixing of the bladder contents. The first trace of fluorescence in the washings was then easily seen in ultraviolet light.

The injection of dextran (intravenously) and creatinine (intravenously or subcutaneously) was made when the urine flow had attained a high steady level.

A typical example of an experiment is given below:

14.7.53 Dog: K/3 Body weight: 26.4 kg.

Time

0 10 ml pentobarbital sodium intravenously, followed by a continuous intravenous infusion of physiological saline at a rate of about 2 ml /min. Catheter introduced into the bladder and the urine flow is measured at regular intervals.

45 10 ml 10 % creatinine solution subcutaneously.

60 40 ml 15 % dextran solution intravenously.

75 Blood specimen I.
The bladder thoroughly washed and emptied.

77 First urine collection period begun.

85 Blood specimen II.
Bladder wash-out.
87 Urine period I ended.
96 Blood specimen III.
Bladder wash-out.
98 Urine period II ended.
99 0.5 ml fluorescein sodium (20%) intravenously.
First fluorescence appeared in urine after 1 min. 45 sec.

Following this general schedule a total of six clearance experiments were made in two dogs, comprising a total of nine clearance periods.

In order to obtain a plasma dextran content that was as representative as possible with respect to the urine collection period, these periods were made as short as possible. Equal parts of serum from the blood samples drawn in the beginning and at the end of each urine collection period were then mixed and analyzed.

The serum mixtures and urine specimens were analyzed for creatinine content according to the method of Folin and Wu (1919) and total dextran content by the anthrone method. The dextran from each specimen was then isolated as described in Chapter 3 and the distribution of the different molecular weights was determined according to the turbidity titration technique. When compiling the results the molecular sizes were divided into five groups of molecular weights with an interval of 4,000; the concentration in serum and urine of each molecular weight group was computed, and finally the renal clearance of each molecular class was calculated according to the equation (22) (page 56) and expressed as a percentage of the simultaneously performed creatinine clearance (= the glomerular filtration rate).

Results

The experimental data and results are tabulated in Table 12. It is found that the most low-molecular dextran in the preparation used with a representative mol. wt. of 8,500 has a clearance value that averages 90.9% of that of creatinine, and the mol. wt. 12,500 — 71.7%, 16,500 — 48.7%, 20,500 — 28.7%, 24,500 — 16.3%, and 28,500 — 11.0%, all expressed as percentages of the simultaneously performed exogenous creatinine clearance. The clearance values in each group show a relatively wide variation. However, the mean figures can be assumed to be representative for the different groups of molecular sizes.

When comparing the experiments where no anaesthesia was used with those where pentobarbital sodium was applied as intravenous injections, no significant difference in the clearance values can be observed.

Discussion

In experiments of the type reported here, which involve so many different methods of analysis and calculations, the experimental error must

Table 12. *Renal clearance of dextran and its relation to molecular weight in normal dogs.*

Dog	Length of period min.	Creatinine			Dextran Molecular weight																	Total plasma conc. mg %	Anaesthesia
					8,500 6,500–10,500			12,500 10,500–14,500			16,500 14,500–18,500			20,500 18,500–22,500			24,500 22.500–26,500			28,500 26,500–30,500				
		Excretion mg/min.	Plasma conc. mg %	Clearance ml/min.	Excretion mg/min.	Plasma conc. mg %	Clearance in % of $C_{creat.}$	Excretion mg/min.	Plasma conc. mg %	Clearance in % of $C_{creat.}$	Excretion mg/min.	Plasma conc. mg %	Clearance in % of $C_{creat.}$	Excretion mg/min.	Plasma conc. mg %	Clearance in % of $C_{creat.}$	Excretion mg/min.	Plasma conc. mg %	Clearance in % of $C_{creat.}$	Excretion mg/min.	Plasma conc. mg %	Clearance in % of $C_{creat.}$		
St. I	15	0.336	6.35	53.0	53.9	112	91.0	28.8	72.0	75.5	11.7	40.0	54.9	5.52	28.0	37.1	2.45	20.0	23.0	1.22	13.0	17.7	328	—
II	18	0.333	5.85	57.0	20.8	40.0	91.2	12.1	29.0	72.8	5.15	19.0	47.6	2.57	18.5	24.4	1.29	17.0	13.3	0.64	14.0	8.1	201	—
St. I	21	0.338	6.25	54.0	14.1	28.5	91.1	6.54	15.5	78.2	3.19	12.0	49.3	2.30	12.0	35.3	1.11	9.5	21.7	0.48	9.0	9.8	169	—
St. I	16	0.365	7.02	52.0	9.67	20.5	90.8	4.40	12.5	67.8	2.32	9.5	46.9	1.08	8.0	26.0	0.49	6.5	14.6	0.25	5.0	9.6	87.5	+
K. I	15	0.550	5.55	99.2	60.7	63.0	96.8	31.5	40.5	78.3	11.5	22.0	52.3	3.73	15.0	25.0	1.26	9.0	14.1	0.74	6.0	12.4	170	+
II	15	0.577	6.38	90.5	29.4	37.0	87.8	14.4	24.0	66.3	7.70	17.0	50.0	2.61	11.0	26.2	1.04	9.0	12.7	0.65	7.0	10.3	131	+
K. I	10	0.480	4.90	98.0	67.1	72.0	94.8	27.6	41.0	68.8	8.70	20.0	44.5	3.95	15.0	26.9	1.58	8.0	20.1	0.63	6.0	10.7	183	+
K. I	10	0.345	4.06	85.0	41.6	58.0	84.6	18.7	34.0	65.0	5.73	16.0	42.3	1.77	9.0	23.2	0.83	8.0	12.2	0.52	6.0	10.2	149	+
II	11	0.300	3.90	77.0	27.2	40.0	88.4	12.3	22.0	72.3	4.85	12.5	50.5	2.22	8.5	33.8	0.63	5.5	14.8	0.32	4.0	10.3	106	+
					Mean		90.9	Mean		71.7	Mean		48.7	Mean		28.7	Mean		16.3	Mean		11.0		

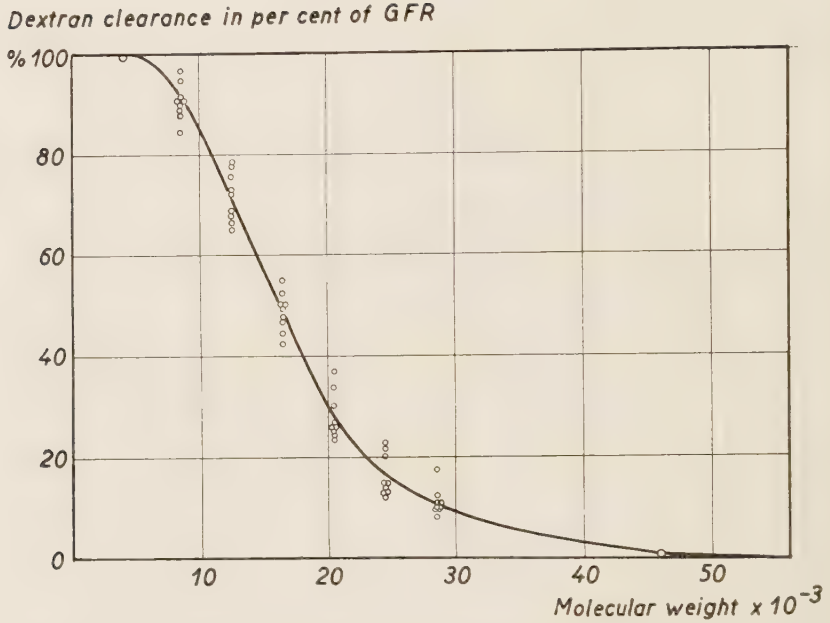

Fig. 20. The renal clearance of dextran (in per cent of the glomerular filtration rate) and its relation to the molecular weight of the dextran molecules, in normal dogs.

necessarily be relatively large. Furthermore, the variability which occurs in all biological materials also affects the experimental data. Nevertheless, the average figures obtained may be supposed to be representative, and thus serve as a basis for discussing the mechanism of the passage of large molecules through the glomerular capillary wall and the physical structure of this membrane.

Obviously, the dextran preparation used here had a molecular weight distribution slightly too narrow to cover the whole "spectrum" of different filtration rates. However, nothing prevents the inclusion of the experimental data on the clearance of a low-molecular dextran fraction (page 68) and the data on the largest molecular weight detectable in the urine (page 61), because the same dogs were used in these experiments.

It will thus be possible to construct Fig. 20, where the filtration rates of the different molecules relative to that of creatinine are plotted against the molecular weights. From this curve it appears that low-molecular dextran (mol. wt. of about 6,000 to 7,000) will pass into the urine as easily as creatinine or water, and at higher molecular weights there will be an increased restriction to their passage through the kidney.

The curve approaches the base line more or less asymptotically, and at a molecular weight of about 50,000 the clearance values are very close to zero.

Thus, a complete scale of filtration rates for a series of identical molecules differing only in molecular weights is obtained.

Most of the data in the literature dealing with this problem have been obtained by the use of chemically non-identical substances, as a rule proteins of different origins. In such a case several variables affect the passage of the molecules through the kidney. The form, structure, degree of solvation, electrical charge, etc. of the molecules used, will all affect the results. The tubular activity, which may vary for different substances, will likewise make the results difficult to interpret. Consequently only indirect information on the relationship between molecular magnitude and penetrability of the membrane will be obtained.

The only attempts to elucidate this question by the use of polymolecular substances have been those of Hoffman and Kozoll (1946), using gelatin fractions in humans, Shaffer, Critchfield and Carpenter (1948) with polyethylene glycol fractions in the dog, and Brewer (1951) using dextran fractions in rabbit experiments. These authors have estimated the renal clearance of various more or less narrow fractions of the substances mentioned and then compared the clearance values obtained with the average molecular weights of the fractions.

However, from the discussion in the beginning of this chapter it appears that the apparent clearance value of a polydisperse fraction, the molecules of which are subject to various restrictions to their passage through the kidney, is composed of the contributions of the clearances of each molecular species and is a rather complex function of the time that has passed after the injection. This time dependence varies with the composition of the fraction used. Such clearance experiments with polymolecular fractions will therefore give uncertain results even if the same time schedule is followed in the different experiments.

Nevertheless, in the experiments of Brewer (1951) in rabbits the clearance values for three different dextran fractions correspond rather well with the clearance curve for dogs here reported.

Further discussion of these results will be given in Chapter 5 below.

CHAPTER 5

General Discussion

General Criteria of a Clearance Test Substance

Smith (1951) summarizes the requirements that a standard substance must fulfil to make it suitable for measuring the glomerular clearance, in the following five items:

(1) It must be completely filterable at the glomerulus; i.e. its molecular size must not be so great as to prevent its passing through the glomerular membrane, and it must not combine with or be absorbed by the plasma proteins.

(2) It must not be synthesized or destroyed by the tubules.

(3) It must not be reabsorbed or excreted by the tubules.

(4) It must be physiologically inert, so that its administration does not have any disturbing effect upon the body, and particularly upon the kidneys.

(5) It must be of such a nature that its concentration in plasma and urine can be determined accurately.

These five items will now be considered with respect to the use of dextran in molecular "sieving" experiments.

Item 1.—Test substances for the study of molecular sieving through the glomerular capillary wall must fulfil all the above requirements except the first. Such a substance must have molecular dimensions of the same order of magnitude as the presumptive "pores" in the membrane so that any restriction of their passage will be measurable. A dextran preparation having any desired composition of molecular weights can easily be prepared by appropriate degradation and fractionation procedures. Control experiments have demonstrated that no measurable degree of combination between dextran and plasma protein exists.

Item 2.—There is no evidence that dextran can be synthesized or broken down by the tubular cells.

Item 3.—In Chapter 4 it is shown that dextran is not excreted nor reabsorbed by the tubules to any degree that can be measured by the clearance technique.

Item 4.—The use of dextran in intravenous infusions to prevent or combat shock in surgical patients has demonstrated that only in exceptional cases has the administration of this substance had any untoward effects upon the body. With the dextran preparation here used no

reactions of this character have been observed with the exception of the rat experiments discussed in Chapter 4. No significant transpositions of water within the body may be obtained with the doses of dextran used in these experiments. It has been shown by Fleming and co-workers (1952), that dextran injection may raise the glomerular filtration rate and the renal plasma flow an insignificant degree. This need not be considered here, because the dextran clearance is always given as a percentage of the glomerular filtration rate and no absolute figures are used.

Item 5.—The absolute concentration of dextran as well as its relative molecular weight distribution can be determined with sufficient accuracy in blood as well as in urine (see Chapter 3).

Besides the magnitude of the molecules just discussed other factors such as their shape, electrical charge and other characteristics may influence their suitabiliy as a test substance in this respect.

An advantage of using a neutral polysaccharide such as dextran for a test substance is that the molecule has no electrical charge that can interfere with the membrane either in the form of adsorption or as a kind of repulsion.

According to Ingelman and Halling (1949) the dextran molecules are more or less oblong and thread-like. There is reason to believe that the molecules when being dissolved in water are coiled at random. Ogston and Woods (1953) recently confirmed this assumption. By considering sedimentation and viscosity data for dextran they found that the axial ratio must be very close to unity, therefore they conclude that dextran molecules in solution are highly swollen and not very asymmetric. Furthermore, the observations made in the present work on the relationship between intrinsic viscosity for this type of dextran and molecular weight (Chapter 3, page 19) and the solubility characteristic $f(M)$ and the molecular weight (Chapter 3, page 31) agree with the conclusions of Ogston and Woods (1953). The dextran molecules together with their solvation water envelope can therefore be regarded as more or less spherical particles.

The possibility of un-coiling and orientation of the molecules in a streaming velocity gradient should be discussed. No information on the behaviour of dextran in this respect, as for instance measurements of its viscosity at different rates of shear, has been found in the literature. Considering the lack of anomalous viscosity of any measurable degree in the molecular weight range and the concentrations here investigated, and the conclusions of Ogston and Woods (1953) it seems, that a stretching and orientation of the dextran coils will be effected only at very high rates of shear, which presumably will never occur in a biological system.

In experiments on the transport of molecules over the capillary membrane Pappenheimer and coworkers (1951) have demonstrated that the

most usable parameter of a molecule in such connections is the "effective diffusion diameter", that is, the diameter of a sphere with the equivalent diffusion coefficient (according to Einstein-Stokes). This measure will be used in the discussion that follows. Fig. 3 (page 21) demonstrates the relationship between the molecular weight of the dextran used in the experiments here reported, the diffusion coefficient and the Einstein-Stokes radius of the molecules.

The Clearance Technique

The author used single injections of the test substance and peripheral venous blood specimens in these experiments. Some objections have been made to the use of such a technique.

Brun, Hilden and Raaschou (1949) compared this technique with a continuous infusion of the test substance, thus attaining a relatively constant concentration level in the blood. They found, when using the single injection technique, that an error is introduced which results in apparent clearance values that are erroneously low. This is particularly so when the test substance has a high renal extraction ratio, as e.g. diodrast and para-amino hippuric acid. The cause of this discrepancy is the arterio-venous concentration difference, which for diodrast can amount to about 30%. With inulin this difference is only about 7%. Consequently the results for inulin clearance compare relatively well for both techniques. This must also apply to the use of dextran, which like inulin has a low extraction ratio, thus resulting in negligible arterio-venous concentration differences.

On the other hand, dextran is a polymolecular substance with a series of molecules, some of which will accumulate within the blood stream during a continuous infusion. The rise in the blood concentration of these large molecules, and the simultaneous decrease of the low-molecular fraction will create difficult problems when calculating the mean plasma concentration for the clearance periods. The single injection method seems to be the best in these molecular "sieving" experiments, and short clearance periods are to be preferred. Furthermore, the precaution of drawing blood specimens at both the beginning and the end of the periods with subsequent mixing of equal parts of these samples will make the results more reliable.

Relationships between the Size of the Penetrating Molecule and the Structure of the Membrane

A great amount of literature concerning the different aspects of permeability phenomena is available. For a comprehensive review the reader is referred to Teorell (1949) and Davson and Danielli (1952). However, most work has been directed to the problem of electrolyte permeability of cell membranes and similar questions, while only scattered data are to be found on the permeability of membranes to large molecules and molecular sieving phenomena. Brief mention will here be made of the most significant contributions in this field.

Thus Manegold and co-workers have in a long series of papers discussed the characteristics of artificial membranes and their sieving effects on polydisperse particles (Manegold and Hofmann, 1930). Elford (1935) approached these problems in a similar manner, and Ferry (1936) derived an expression for the retardation of particles streaming through narrow pores. Grabar (1943) made use of the sieving action of porous membranes for the fractionated ultrafiltration of biological fluids. The closely related question on diffusion of non-electrolytes in gels has been treated by Friedman and Kraemer (1930). The pure geometrical steric hindrance factors acting in such systems have been discussed by Svedberg and Rånby (1945).

At the present time there is no general theory that accounts for the restricted filtration of non-electrolytes across a membrane with openings of molecular dimensions. However, Pappenheimer, Renkin and Borrero (1951) have recently approached a similar problem and have deduced an expression for the restricted diffusion of molecules through submicroscopic pores. Their theory utilizes a probability deduction similar to that of Svedberg and Rånby (1945) and a factor for the viscous drag of the pore walls (Ladenburg, 1907). Experimental data for molecules ranging in size from sodium chloride to hemoglobin have been obtained in perfusion experiments on isolated hindlimb preparations from cats, and these data agree rather well with their theoretically deduced formula.

By considering the relationship between pressure and hydrodynamic flow across the capillary membrane in these preparations they find that the fractional pore area available for passage of a molecule the size of water is less than 0.02%, while the same area for the glomerular membrane computed from data in the literature should be about 1-2%, of the total filter area.

By comparing the area available for the passage of molecules of different sizes they conclude that a Gaussian distribution of pore radii with a mean radius of 24 Å and a standard deviation of 12 Å is the broadest distribution of pore sizes consistent with their observations. If uniform cylindrical pores are assumed, their radii should be 30 Å.

Furthermore they find that the transfer over the capillary membrane by diffusion is large when compared to that of filtration.

In a recent paper Chinard (1952) deduced an expression for the formation of glomerular fluid that is in variance with the common concept of glomerular "filtration". By strict thermodynamic considerations he derives an expression for the transport of water across the glomerular membrane, and he finds that a diffusion process and not a filtration process is compatible with these deductions. By tentative calculations following this concept he finds that the collective area involved in the passage of water across the glomerular membrane should have a magnitude of 2.1 m^2, a figure that compares rather well with the anatomically measured area of 1.56 m^2 (Vimtrup, 1928). Chinard visualizes the glomerular membrane as a gel-like structure, the whole area of which is involved in the passage of water. The rate of net passage of a substance across this membrane should be proportional to the diffusivity of this substance in the wall structure. This "intramural" diffusivity of a low molecular compound may be close to its diffusivity in water, while for a more high molecular substance the diffusivity in the wall may be so small as to be negligible.

From this it may be seen that no definite knowledge about the nature of the glomerular "filtration" process is yet available and that the question of the ultramicroscopical physical structure of the glomerular membrane is far from solved.

How do the data obtained in the dextran clearance experiments compare with the theoretical deductions just discussed?

First it can be stated that there is no sharp limit as to the passage of dextran molecules across the glomerular membrane. It is found that a slowly increasing restriction to passage begins at a molecular weight of about 6,000, when dextran molecules are used as a measuring tool, and at a molecular weight of 30,000 the rate of passage is still about 10% of that of the low molecular components (Fig. 20). Considering the asymptotical approach to the base line of the curve it seems possible, that even very large dextran molecules could be found in the urine, if a method with sufficient sensitivity was available. This agrees with the observation of Campbell and co-workers (1953) in their polyvinyl pyrrolidone experiments, where molecular weights of 100,000 were found in the urine.

Following Pappenheimer (1953), if we make use of the Einstein-Stokes radius as a parameter of the size of the molecules (data given in Fig. 3, page 21), it is found that the first molecule subjected to restriction of the glomerular membrane has a radius of about 18 Å, and that molecules with such radius as 50 Å still can pass into the urine. If we visualize the membrane as being a porous plate this seems to indicate that the pores have a distribution of different sizes, the smallest of such dimensions that molecules with radii less than about 18 Å pass without

interference, while only a few pores are large enough to permit the passage of the large molecules. This is not necessarily the case. If the pores are of uniform size the restriction to the passage of the molecules will increase the more their dimensions approach those of the pores. Thus the result in this case will be similar to that which would be the consequence of a pore size distribution.

Unfortunately the knowledge of the nature of the glomerular membrane is yet too fragmentary to permit any conclusions as to how the dextran clearance data fit into such a system as that derived by Chinard (1952). Much experimental work remains to be done utilizing polymolecular as well as homogeneous substances, and studies of their diffusion and filtration through well-defined artificial membranes and gels as well as through living animal membranes must be carried out before this problem can be answered definitely.

Finally, some comparisons will be made between the size of the dextran molecules found in the urine, and the size of some proteins. If the restriction to passage across the glomerular membrane was a function of the size of the molecules only, we would expect that albumin (diffusion radius 35 Å) and hemoglobin (31 Å) would pass through the membrane rather freely. This is however not the case, and by considering the electrical charge and other factors it seems conceivable that the proteins are more restricted in their passage than are electrically neutral molecules such as dextran. Consequently, dextran molecules rather than protein molecules will thus more closely depict the physical openings of the membrane, if the pore theory is assumed.

In Fig. 17 (page 63) which shows the largest dextran molecule that could be detected in the urine, the critical limit above which proteinuria occurs, was at a molecular weight of about 55,000. The effective radius for such a molecule is about 50 Å, while the radius of the simultaneously penetrating albumin molecule only is 35 Å. These molecules should thus possess a relatively comparable penetrability, and this further supports the view that protein molecules are more restricted in their passage through the glomerular membrane than are dextran molecules of equal size.

Concluding Remarks

Although the principle for the formation of glomerular fluid is supposed to involve only passive physical processes, it is quite obvious from the above discussion that this important physiological mechanism is rather incompletely understood. Much evidence indicates that the common physical laws, which govern fluid movements in macroscopic systems, do not hold in ultramicroscopic structures of molecular dimensions. Thus the law of Poiseuille for laminar flow does not seem to be applicable (see e.g. Duclaux and Errera, 1926), and no modification to account for this discrepancy has yet been derived. Similarly, the laws for friction and viscous drag do not necessarily apply in these systems. Here also much experimental work has to be done to elucidate these questions.

However, it is felt that a technique such as that outlined in this study may be useful in investigating different physiological membranes and their characteristics in normal and pathological conditions. Its character of a micro-method makes it applicable even if limited amounts of filtrate are available, as is the case with some extravascular fluids, such as lymph, edema fluid, burn blister contents, etc. Furthermore, an extensive investigation of the excretion of dextran in different proteinuric conditions may clarify the different responsive mechanisms.

General Summary

The purpose of this investigation has been to find a way to characterize some of the qualities of an animal membrane. The experimental work in this study has been directed to the problems of the glomerular membrane and its function.

The principle used has been the following. A polymolecular dextran solution with a selected molecular weight distribution is used as a clearance substance. By analysis of blood and urine, quantitatively as well as qualitatively with respect to the molecular weight distribution in these two fluids, information on the relative filtration rates of the different molecular sizes has been obtained.

In Chapter 1, a short survey of the literature dealing with the mechanism of glomerular fluid formation and related problems is given. The more important contributions to the study of the filtration of macromolecular compounds by the kidney are referred to.

In Chapter 2 the problems are defined and the general principle of the investigation is outlined.

The methods that have been used are described in Chapter 3. First, a general description of the character of dextran, with special regard to the dextran preparations and fractions used, is given. By comparing the data obtained for viscosity and molecular weight of narrow dextran fractions a general relationship between these two characteristics is found, which corresponds to an expression derived by Mark (1938). Data for the diffusion coefficients and effective diffusion diameters (Einstein-Stokes) for dextran of different molecular weight are given.

The anthrone method has proved suitable for the quantitative analysis of dextran in serum and urine. A modification of this method is described together with a method that has been developed for the quantitative recovery of dextran from these fluids, yielding isolates where no change in the size distribution of the dextran preparation is inferred.

For the determination of the molecular weight distribution in dextran samples, a method has been developed based on the principles of Schulz (1937) for the fractionation of high polymers and utilizing the principle of turbidimetric titration, given by Morey and Tamblyn (1945). A modified turbidimeter according to the principles of Stein and Doty (1946) and Hadow, Sheffer and Hyde (1949) was constructed. Using this apparatus, the relations between the precipitation point and the concentration of polymer, the concentration of precipitant (alcohol), and

the degree of polymerization have been investigated. By applying a deduction according to Morey and Tamblyn (1945) a system was developed, by which the relative distribution of molecular weights in an unknown dextran preparation can be calculated with a fair degree of accuracy. The quantitative relationship between the turbidity of the turbid suspension and the weight of the precipitate is investigated for different molecular sizes and an empirical correction procedure is applied to compensate for the deviation from linearity found.

The reproducibility of the method is tested in a series of experiments, and it is found that the standard deviation of the molecular weight figures obtained is about 5%, while the figures for the relative concentrations vary from 5 to 8%. Furthermore, some control experiments are performed demonstrating results achieved by different techniques.

In the discussion of Chapter 3 further details of the procedure are given and the influence of the distribution of the dextran preparations on the results is discussed. The problems of "reverse order" precipitation are summarized.

Chapter 4 deals with the physiological experiments that have been performed in humans and experimental animals. First, a series of human experiments were performed elucidating the excretion of dextran via the kidney as investigated by clearance technique. The dynamics of the excretion of polydisperse substances is discussed and some comments on the behavior of inulin in this respect are given.

The maximal molecular weight that can be detected in the urine of different species after dextran administration has been investigated. It is found that under normal conditions there exists an upper limit for the penetration of macromolecules in detectable amounts. This limit varies slightly for different animal species. Above this limit the restriction to the passage of the molecules prevents their passage into the urine in demonstrable quantities. In proteinuria this limit is raised, and it is found that the more protein there is in the urine, the larger will be the dextran molecules found in the urine. The aberrant behavior of the rat species with regard to dextran injections is discussed.

The renal clearance of a low-molecular dextran preparation has been investigated in normal dogs. It is found that, although histological evidence indicates a tubular reabsorption of dextran, this reabsorption is quantitatively of such a low order of magnitude, that the clearance technique applied fails to demonstrate this action of the tubules. It is concluded that this influence of the tubules, presumably caused by passive diffusion, is less pronounced for larger dextran molecules and that this interference may have no demonstrable influence on the filtration rates reported.

Finally, a series of dog experiments was performed in which the relative filtration rates of different molecular sizes of the dextran preparation used were measured and compared to the simultaneously per-

formed creatinine clearance. It was found that dextran molecules with molecular weights less than 6,000 to 7,000 pass through the glomerular membrane without any demonstrable restriction. For larger molecules there is a continuously increasing restriction as the molecular weight increases, so that the clearance curve more or less asymptotically approaches the base line.

The last chapter discusses the experimental results in relation to the various theories for the function of the glomerular membrane.

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